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Determination of the coumarin derivative cloricromene acid in rabbit plasma and platelets☆

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ABSTRACT

Two methods for the determination of cloricromene acid in biological samples are described. Cloricromene acid is a catabolite of cloricromene, a coumarin derivative which is active in the cardiovascular system. After oral administration of cloricromene to a rabbit, plasma and platelets were taken at different times and cloricromene acid was then isolated by solid-phase extraction with Sep-Pak C_{18} cartridges using acetonitrile–tetrahydrofuran–20% aqueous acetic acid (15:11:74, v/v/v) as eluent. The analyses were performed by reversed-phase high-performance liquid chromatography (RP-HPLC) combined with fluorescence detection with excitation at 310 nm and emission at 390 nm. The limit of quantification by RP-HPLC was about 50 pg. The catabolite in the plasma was identified by continuous-flow fast atom bombardment mass spectrometry (CF-FAB-MS), also used as a complementary means of RP-HPLC determination. The results obtained by RP-HPLC and CF-FAB-MS showed good agreement.

INTRODUCTION

Cloricromene, a coumarin derivative (Proendotel Sinax; Fig. 1a), is a drug with pharmacological effects on the vessel wall and on platelets, where it inhibits aggregation and release of β-thromboglobulin, platelet factor 4 and thromboxanes under a variety of experimental conditions [1–4]. Recently, a further study has demonstrated that cloricromene inhibits the release of free arachidonic acid derived from glycerophospholipids (precursors of thromboxane, prostaglandins and leukotrienes) through its blocking action on phospholipase A2 [5]. It has been also found either *in vitro* or *in vivo* that cloricromene (Fig. 1a) in the blood is rapidly metabolized into a stable catabolite (Fig. 1b) through the hydrolysis of an ester bond within the molecule [6].

A method for the determination of cloricromene acid in human plasma and platelets by high-performance liquid chromatography (HPLC) has been described by Travagli *et al.* [6]; however, the approach is considered to be unsuitable for the routine assay of cloricromene

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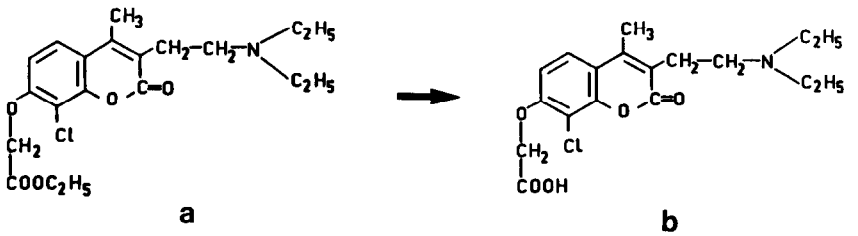

Fig. 1. Structures of (a) cloricromene and (b) cloricromene acid.

acid, owing to the lack of an accurate and sensitive determination of the catabolite.

In order to develop a complete procedure for the determination of cloricromene acid from rabbit plasma, we applied solid–liquid extraction and reversed-phase (RP) HPLC coupled with fluorimetric detection. Positive-ion continuous-flow fast atom bombardment mass spectrometry (CF-FAB-MS) was also used not only for the identification of cloricromene acid, but also as a complementary means for the determination of this catabolite.

EXPERIMENTAL

Reagents and chemicals

HPLC-grade reagents, purchased from Rudipont (Hetalab, Parsipanny, NJ, USA), were used for sample extraction and for HPLC and CF-FAB-MS analyses. Cloricromene and cloricromene acid were obtained from our laboratories.

Preparation of plasmatic and platelet samples

Blood samples were obtained from three conscious male New Zealand rabbits at various times after oral administration of 100 mg of cloricromene (Proendotel). The blood was rapidly mixed with 3.8% sodium citrate (9:1, v/v) and then centrifuged at 150 *g* for 10 min to obtain platelet-rich plasma (PRP). The PRP was further centrifuged with a Microfuge (Eppendorf, Hamburg, Germany) to separate plasma from platelet pellet.

Extraction of cloricromene acid and recovery experiments

The plasma (0.5 ml) was diluted with water (1:1) and then applied to a C_{18} cartridge (Sep-Pak C_{18}; Waters, Milford, MA, USA), which had previously been washed with 2 ml of acetonitrile and 2 ml of water. After removing hydrophilic impurities and salts with 3 ml of water, cloricromene acid was isolated from proteins that remained in the cartridge with a mixture (5 ml) of acetonitrile–tetrahydrofuran–20% aqueous acetic acid (15:11:74, v/v/v).

A 1-ml volume of this extract was used for HPLC analysis. The remaining aliquot (4 ml) of the solution was dried with a SpeedVac concentrator (Savant Instruments, Farmingdale, NY, USA) and the dried sample was dissolved in methanol–water–glycerol (75:23:2, v/v/v) containing 5 ng/μl internal standard (cloricromene) for CF-FAB-MS analysis.

The platelet pellet was mixed with 1 ml of acetonitrile and then sonicated in an ice-cold bath for 30 s. The solution was applied to a Sep-Pak C_{18} cartridge that had previously been washed with 2 ml of water and 2 ml of acetonitrile. After washing with 3 ml of acetonitrile, cloricromene acid was eluted with 1 ml of the same eluent as used for the acid extraction from plasma. A 50-μl volume of this extract was used for the determination of cloricromene acid by RP-HPLC.

In order to test the recovery of cloricromene acid from plasma and platelet, two kinds of experiments were conducted. In the first experiment, different amounts of cloricromene were added to a PRP from an untreated rabbit for 10 min at 37°C to evaluate the overall recovery of the acidic catabolite from PRP. In fact, under these conditions cloricromene is completely metabolized to the acid [6]. After the incubation PRP was processed as described above.

In the second experiment, different amounts of

cloricromene acid (0.05, 0.1, 0.5, 2.5 and 12.5 nmol) were added to both the plasma and platelet pellet from an untreated rabbit in order to determine the relative recovery of the acid from both biological samples. These extracts were analysed by RP-HPLC (see below).

HPLC assay

Cloricromene acid was separated on an RP-HPLC column (μBondapak C_{18}, 10 μm; 30 cm × 3.9 mm I.D.) (Waters, Milford, MA, USA) with a guard column (Spheri 5, RP-18, 5 μm) (Brownlee, Labs., Santa Clara, CA, USA) and monitored with a Model LS4 fluorescence detector (excitation at 310 nm and emission at 390 nm) (Perkin-Elmer, Norwalk, CT, USA).

The flow-rate was mantained at 1 ml/min (Waters Model 510 HPLC pump) using a mobile phase of 0.005 *M* potassium phosphate buffer (pH 3)–acetonitrile–tetrahydrofuran (74:15:11, v/v/v).

The extracts (50 μl for each injection) were introduced into the chromatographic system by a Wisp 710 autosampler (Waters). The peak area corresponding to cloricromene acid was calculated with a Chromjet integrator (Specra-Physics, San Jose, CA, USA).

Continuous-flow fast atom bombardment mass spectrometry

CF-FAB-MS was performed on a VG-7070 EQ mass spectrometer (VG Analytical, Manchester, UK) with a CF-FAB ion source and PDP-8 data processing system. A laboratory-made CF-FAB probe (like the VG CF-FAB probe), a μLC-500 micro flow pump (Kontron, Zurich, Switzerland) and a Rheodyne (Cotati, CA, USA) Model 7520 injector with a 0.5-μl internal loop were used.

The mobile phase [methanol–water–glycerol (75:23:2, v/v/v)] was carried into the mass spectrometer by a 1 m × 50 μm I.D. fused-silica capillary column at a flow-rate of 2–3 μl/min. The temperature of the ion source was kept at 25–30°C. Xenon was used as the bombarding gas with a kinetic energy of 8 keV. The resolution was 500. The data for determination of cloricromene acid were recorded using the selected ion monitoring (SIM) mode.

RESULTS

RP-HPLC determination of cloricromene acid

The most critical step in this assay was the isolation of cloricromene acid from proteins and other components that could interfere with the quantitative measurement. In order to choose the optimum isolation conditions, several procedures were tested to extract the acid from plasma and platelets, including liquid extraction with different solvents (acetonitrile, tetrahydrofuran, methanol and the mobile phase itself) and solid-phase extraction. The best result was obtained with solid-phase extraction, using a mixture acetonitrile–tetrahydrofuran–20% aqueous of acetic acid as eluent (Fig. 2).

The recoveries of cloricromene acid added to plasma and platelets were 98% and 85%, respectively. The same values were obtained in experiments in which PRP was incubated with cloricromene. The results indicate that the total amount of cloricromene is converted and recovered as cloricromene acid either in plasma or in platelets. The recovery was also linear over the range of 40 ng–10 μg for 1 ml of plasma and 6–200 pg for 10^6 platelets. The regression equation and linear regression coefficient (calculated from five different concentrations) were $y = 238316x + 2475$ and $R = 0.999$ for plasma and $y = 1370x + 4402$ and $R = 0.986$ for platelets, respectively, where x is the concentration of cloricromene acid in plasma

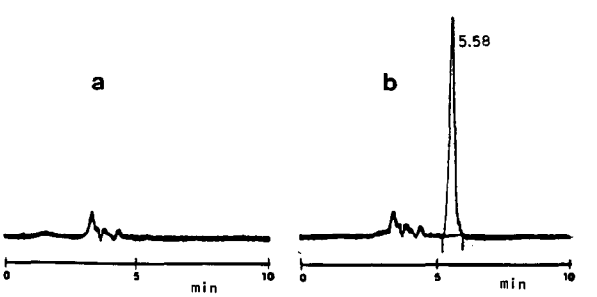

Fig. 2. RP-HPLC separation of cloricromene acid from plasma extract. (a) Blank plasma; (b) plasma from a treated rabbit. Retention time of cloricromene acid = 5.58 min.

TABLE I

VALIDATION PARAMETERS OF METHOD CALCULATED FROM HPLC DATA

Parameter	Standard solution	Plasma	Platelets
Linearity range	50 pg–3 ng (R = 0.9987)	40 ng/ml–10 μg/ml (R = 0.9992)	6–200 pg per 10^6 platelets (R = 0.9862)
Accuracy error	–	−2%	−15%
Precision error (R.S.D.)	–	2.9%	3.5%
Limit of quantitation (L.Q.)[a]	50 pg	40 ng/ml	6 pg per 10^6 platelets

[a] L.Q. = $X_B + 10\sigma_B$, where X_B is the mean height of the noise and σ_B is the standard deviation, according to Long and Winefordner [7].

(ng/ml) or platelets (pg per 10^6 platelets) and y is the peak area of cloricromene acid.

The accuracy of the method was determined. The mean errors (five samples for each concentration) ranged from −5.2% to +1.5% for plasma and from −10.3% to −17% for platelets. The relative standard deviation (R.S.D.) was no higher than 3% for plasma and 4% for platelets. Table I shows the mean validation parameters calculated for the method. Repeated analyses of the same sample showed an intra-day variability of 4% and a day-to-day variability of 7% determined on seven different days.

In order to identify the peak measured in plasma by RP-HPLC, the extracts were analysed by CF-FAB-MS. Fig. 3 shows the positive-ion CF-FAB mass spectrum of the purified sample. The ion at m/z 368 corresponds to the protonated molecular ion of cloricromene acid.

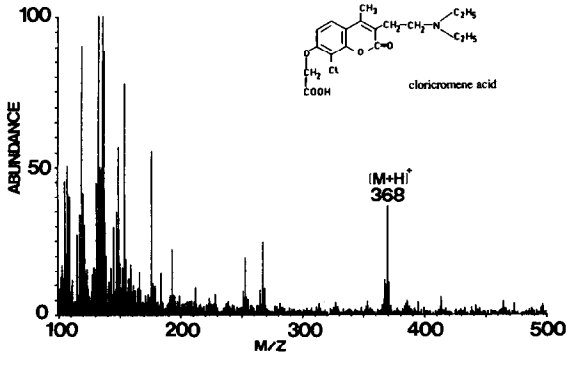

Fig. 3. Positive-ion CF-FAB mass spectrum of cloricromene acid (relative molecular mass 367.5) extracted form rabbit plasma.

CF-FAB-MS determination of cloricromene acid

CF-FAB-MS [8] has been used for pharmacological studies of various drugs [9–11]. The use of a different but complementary method for the determination of cloricromene acid was applied to verify the RP-HPLC assay. In order to improve the accuracy of the determination by CF-FAB-MS, we used cloricromene itself as the internal standard, as this molecule is not present in the plasma after oral administration [6].

The abundant protonated molecular ions of both compounds appeared at m/z 368 (cloricromene acid) and m/z 396 (cloricromene). By detecting their $[M + H]^+$ ions (Fig. 4), a quantita-

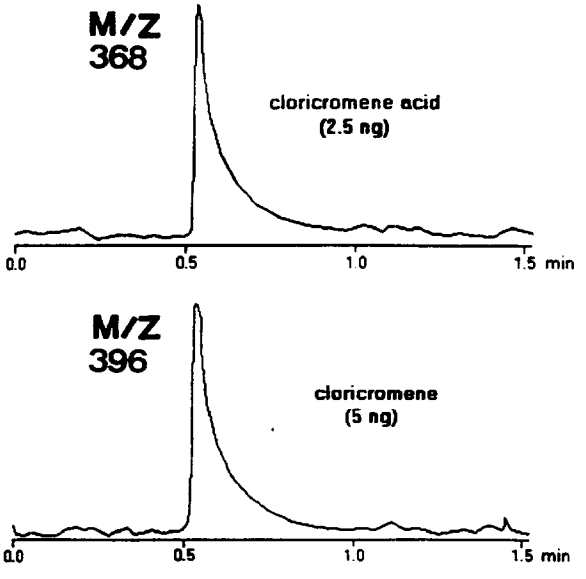

Fig. 4. Selected ion monitoring of cloricromene acid and cloricromene (internal standard).

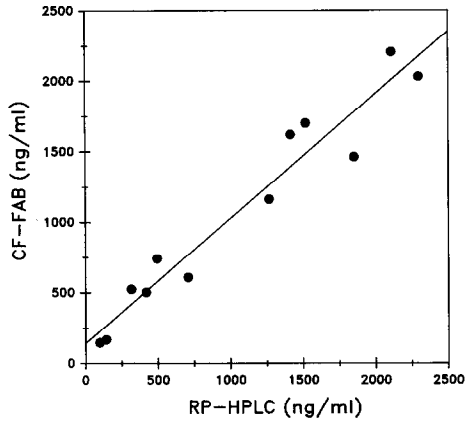

Fig. 5. Correlation between cloricromene acid levels in the plasma measured by RP-HPLC and CF-FAB-MS.

tive assay for cloricromene acid was carried out and the calibration graph was found to be linear over the range 1–25 ng. The linear equation obtained was $y = 0.037x + 0.073$ ($R = 0.997$), where x is the absolute amount of cloricromene acid injected and y is the cloricromene acid/I.S. peak-area ratio.

However, for the accuracy of the determination of the acid in rabbit plasma, the range of cloricromene acid concentration used was between 5 and 25 ng. CF-FAB-MS determination of cloricromene acid using the internal standard is in close agreement with that of the RP-HPLC method. Fig. 5 shows the correlation between cloricromene acid levels in several plasma samples from three treated rabbits measured by RP-HPLC and CF-FAB-MS. The points are distributed around a straight line ($R = 0.969$).

Selected ion monitoring of the protonated molecule of the acid gave a limit of detection of 500 pg.

DISCUSSION

The pharmacological effects of cloricromene on the cardiovascular system are that it dilates dog coronary vessels [12], increases prostacyclin release from vascular cells and affects platelet aggregation, secretion and morphology in normal and pathological conditions [2–4]. It also has a protective effect in a model of coronary artery thrombosis in dog [13].

The present procedures are useful for the determination of cloricromene acid in rabbit plasma and platelets. The preparation of the samples is relatively rapid and the extraction of the acid using Sep-Pak C_{18} gives reproducible results with a high recovery. We confirmed the presence of cloricromene acid in plasma where the drug is metabolized to this stable catabolite [6].

The method, based on RP-HPLC combined with fluorescence detection, is ten times more sensitive than CF-FAB-MS. This is the reason why it was not possible to determine the acid in platelets by CF-FAB-MS, as the levels of cloricromene acid are too low to be detected.

The amount of cloricromene acid in the extracts isolated from plasma was measured by CF-FAB-MS in the SIM mode. Because of the high yield of the recovery from plasma (98%), the internal standard (cloricromene) was added after the extraction step. This avoids cross-reactivity of cloricromene with the substances in the plasma.

Although a chromatographic separation of cloricromene acid is lacking for the CF-FAB-MS determination, the use of Sep-Pak C_{18} for sample clean-up is necessary for this assay. However, the minimum detection levels for the assay of cloricromene acid in biological samples is over 5 ng. The data obtained by this method show good agreement with those obtained by the RP-HPLC procedure ($R = 0.969$).

The results also suggested that none of substances that interfere with RP-HPLC analysis are present in the extracts isolated by solid-phase extraction in spite of the fact that peaks related to the impurities could be observed in the positive-ion CF-FAB mass spectrum (Fig. 3).

Further studies regarding the pharmacokinetic behaviour of cloricromene acid in the body, including its distribution in different tissues, are in progress. These will allow us to compare different formulations of the drug based on the acid levels in the body, in order to find the best one in terms of bioavailability.

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