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Short Communication

Determination of boldine in plasma by high-performance liquid chromatography

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ABSTRACT

A sensitive method for the determination of boldine in blood plasma is described. The procedure involves a direct pH-buffered chloroform extraction of boldine from blood plasma, followed by its assay under isocratic conditions by HPLC with UV detection. The extraction recovery is excellent, and sensitivity and precision of the method are very high, when applied to plasma samples containing pharmacologically relevant concentrations of boldine.

INTRODUCTION

Boldine (Fig. 1) is an alkaloid present in the leaves and bark of boldo (*Peumus boldus* Mol.), a widely distributed evergreen tree native to Chile [1–4]. Boldine-containing boldo leaf extracts and infusions have been used for more than a century in traditional medicine in the treatment of a variety of conditions, amongst which liver complaints and disfunctions are generally mentioned [5,6]. Recently, we reported that chemically pure boldine behaves as a very potent antioxidant in biological systems undergoing lipid peroxidation [7], thus providing a possible rationale for the hepatotrophic properties commonly attributed to boldo. Additionally, boldine has been shown to

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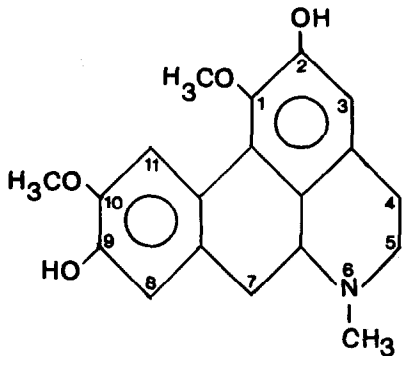

Fig. 1. Molecular structure of boldine (2,9-dihydroxy-1,10-dimethoxyaporphine).

exhibit choleretic [8] and smooth-muscle relaxing properties [9].

The increasing recognition of these pharmacological properties of boldine, plus its remarkably low toxicity and long tradition of human use [5,6,8], have prompted us to undertake the development of an analytical method for its determination in blood plasma.

Previous analytical work based on spectrophotometry [10,11], voltammetry [12] and high-performance liquid chromatography (HPLC) [13–16], provided a basis for the determination of the alkaloid in boldo extracts or in galenic and pharmaceutical boldo-containing preparations. However, none of the previously reported methods appears to be sufficiently sensitive or suitable for the direct quantitative assay of boldine in blood plasma samples. This paper describes a simple and precise method for the determination of boldine in plasma at pharmacologically relevant concentrations.

EXPERIMENTAL

Chemicals and reagents

Boldine was extracted from the bark of *Peumus boldus* with methanol. Following evaporation of the solvent, the residue was partitioned between aqueous acid and chloroform to remove non-alkaloidal constituents. The total alkaloids were re-extracted with chloroform after adjusting the pH of the aqueous phase to 8–9. Boldine was finally isolated from the crude alkaloidal mixture by several recrystallizations from chloroform. The alkaloid (obtained as a 1:1 boldine–chloroform complex) was chromatographically pure [3,11,17], and its identity was established by IR and NMR spectrometry. The melting point of crystalline boldine–$CHCl_3$ was 155–157°C. Boldine crystals and boldine-containing solutions were always kept in the dark and manipulated under indirect light to minimize photolysis. All organic solvents used were HPLC grade.

HPLC assay

Boldine was analysed at ambient temperature (18–22°C) using a system consisting of a Merck-Hitachi pump (Model L-6200) fitted with a LiChrocart LiChrospher C_{18} RP column (E. Merck, Darmstadt, Germany) (12.5 cm × 4.0 mm I.D., 5 μm particle size). A pre-column (4 mm × 4 mm I.D.) of similar features was used to extend the analytical column's lifetime.

Boldine was detected by means of a variable-wavelength UV–VIS detector (Merck–Hitachi L-4200), and the peak areas were quantified with a Merck–Hitachi D-2500 chromato-integrator. The mobile phase, which was always filtered and degassed prior to use, was methanol–0.025 *M* H_3PO_4–0.025 *M* disodium phosphate buffer, pH 7.4 (450:135:415, v/v/v). The final pH of the mobile phase was not adjusted because pH measurements of this water–methanol mixture were found to be imprecise and therefore unreliable. Standard and sample solutions of boldine in methanol were injected (20 μl) and eluted isocratically at a flow-rate of 0.75 ml/min, and detected at 280 nm. The resulting chromatograms for the standards yielded data for the calibration curves.

Extraction procedure

Boldine-spiked plasma samples. Fresh plasma samples (200 μl), obtained from heparinized rat blood, were mixed with 100 μl of boldine-containing 0.2 *M* phosphate buffer (pH 8.2). The latter solution was prepared after dissolving the alkaloid in a small aliquot of 0.1 *M* HCl. The resulting 300 μl of diluted spiked plasma samples were extracted with three consecutive portions of

400 μl of chloroform. The aqueous–organic mixtures thus obtained were vortex-mixed (1 min) and centrifuged in 1.5-ml Eppendorf tubes at 10 000 *g* for 2 min. Subsequently, the lower organic phases containing boldine were recovered by means of a long-needle syringe, and pooled. The collected chloroform extracts were evaporated to dryness under a stream of nitrogen at ambient temperature. The residues were dissolved in 200 μl of methanol and assayed for boldine by HPLC.

Plasma samples from rats given boldine. Fasted rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and administered a single i.v. bolus of boldine (50 mg/kg) as a saline solution. Blood was sampled from aorta at different times up to 30 min, placed in heparinized Eppendorf tubes, and centrifuged at 10 000 *g* for 2 min. Following this procedure, 200 μl of the plasma obtained were mixed with 100 μl of 0.2 *M* phosphate buffer (pH 8.2) and subjected to chloroform extraction as described above.

Statistical analysis

Data are presented as mean ± $S.D._{n-1}$. The equations of the calibration curves were fitted by least-squares linear regression.

RESULTS AND DISCUSSION

The UV spectrum of a solution of boldine in methanol (200 μ*M*) shows three major absorption peaks at 220, 280 and 302 nm (Fig. 2). We selected 280 nm as the working wavelength. At this energy level, the absorbance of boldine is slightly greater than at 302 nm, while at the same time the light absorption by non-aromatic interfering substances, which might be present in the methanol solution obtained after the extraction of the alkaloid from samples, is negligible. Also, the choice of 280 nm should allow the detection of a broad range of hypothetical boldine metabolites, if present in the chloroform extracts.

Fig. 3A illustrates a typical chromatogram of a standard methanol solution of boldine (25 μ*M*) analysed under the conditions described in Experimental: boldine gave a sharp and symmetrical peak with a retention time of 4.3 min. Reproducibility tests for standard solutions showed a variation of less than 3% in the retention time.

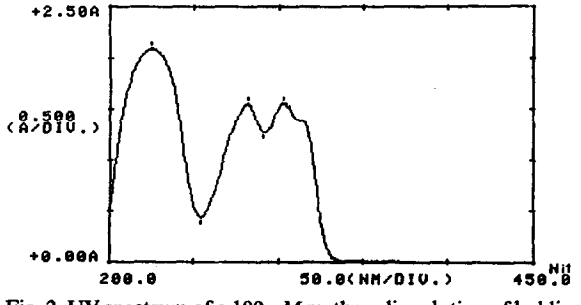

Fig. 2. UV spectrum of a 100 μ*M* methanolic solution of boldine.

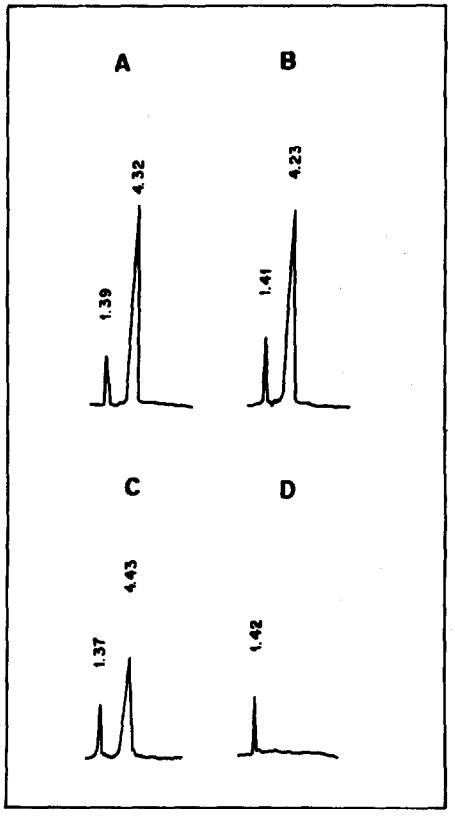

Fig. 3. HPLC chromatograms of (A) a 25 μ*M* standard methanolic solution of boldine, (B) a sample extracted from plasma spiked with boldine to a concentration of 25 μ*M*, (C) a sample extracted from plasma obtained 10 min after injecting a rat with a single i.v. dose 50 mg/kg boldine and (D) a sample extracted from plasma obtained from rats given no boldine. Numbers at peaks indicated retention times in min.

Similar chromatographic behaviour was observed when sample solutions of boldine in methanol were injected into the HPLC column following extraction from either boldine-spiked plasma (25 μM) (Fig. 3B) or from plasma samples obtained after the i.v. application of boldine to experimental animals (Fig. 3C). These results show that, under the general experimental conditions employed here, neither detectable interfering substances from plasma nor boldine-related metabolites (with retention times less than 30 min) are present in the methanol solutions obtained after application of the pH-buffered chloroform extraction procedure.

Finally, the assay of a sample extracted from plasma obtained from rats given no boldine showed only the peak associated with the solvent front (Fig. 3D).

A linear relationship between the peak area in the chromatogram and the concentration (μM) of boldine in standard methanolic solutions was found. The regression equation for the standard curve was estimated to have a slope of 7489, an intercept of −22 971 (area units) and a correlation coefficient of 0.998, for a set of six different concentrations, each assayed in triplicate. The analytical validity of this dependence was established for concentrations from 5 to 75 μM. It is worth noting that pharmacological effects displayed by boldine *in vitro*, such as its antioxidant activity [7] and its smooth-muscle relaxing action [9], are exerted at concentrations in this range.

Table I shows the results of recovery studies in which boldine was extracted from plasma-spiked samples by the extraction procedure described in Experimental. The recovery of boldine was virtually complete (94–107%) throughout the broad range of concentrations studied. The results indicate the absence of an effect of the concentration of boldine on the recoveries. Moreover, the analytical variability of the method decreases as the concentration of boldine in plasma is increased.

The selection of chloroform as the extraction solvent was based on its low cost, easy availability in high purity, and recognized suitability for the extraction and purification of boldine [4].

TABLE I

RECOVERY OF BOLDINE FROM PLASMA-SPIKED SAMPLES

Added[a] (μM)	Recovery (%)	$S.D._{n-1}$	C.V. (%)
5.00	106.5	11.3	10.6
10.0	99.1	5.9	6.0
25.0	93.6	3.1	3.3
50.0	99.6	1.4	1.4
75.0	97.1	1.5	1.5

[a] Each data point represents the average of five individual determinations.

Moreover, chloroform was found to be highly effective in deproteinizing the samples, thus allowing the measurement of total boldine in plasma without the need to use any additional reagent. Aiming for quantitative recovery of boldine from the aqueous phase, the alkaloid was extracted from pH-controlled plasma samples after addition of a phosphate solution buffered at pH 8.2. The latter condition was selected after determining the partition coefficients of boldine at different pH values (not shown). The partition of boldine into the organic phase was significantly higher at pH 8.2 than at the physiological pH of 7.4 (K_p = 31 *vs.* K_p = 23). Although other workers [18] generally employ more basic conditions

TABLE II

ASSAY OF BOLDINE IN PLASMA SAMPLES OBTAINED FROM RATS GIVEN A SINGLE INTRAVENOUS DOSE

Sampling time (min)	Average plasma concentration[a] (μM)
5.0	17.2
10.0	13.9
20.0	11.5
25.0	9.90
30.0	9.60

[a] Each value corresponds to an average of plasma samples obtained from three rats injected with a single intravenous dose of 50 mg/kg boldine. The variation of each estimated value was less than 5%.

for the extraction of alkaloidal compounds, in the present case we limited the pH to 8.2 because it ensured the quantitative extraction of the diphenolic boldine from plasma while it decreased the chances of its auto-oxidation.

The suitability of the extraction method was finally tested on samples of plasma obtained from live rats given boldine. As shown in Table II, the application of the method makes it possible to monitor the time-course of the plasma boldine concentration. The data presented, therefore, demonstrate the potential usefulness of this methodology for future pharmacokinetic studies.

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