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Determination of narciclasine in serum by reversed-phase high-performance liquid chromatography: comparison of amperometric, ultraviolet photometric and fluorescence detection

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ABSTRACT

Narciclasine was determined in the blood of mice by reversed-phase high-performance liquid chromatography, using a C_{18} stationary phase and a mobile phase of methanol–0.025 *M* potassium dihydrogen phosphate (50:50, v/v) of pH 5.5. Amperometric detection at a carbon fibre array working electrode held at +1.8 V (Ag/AgCl) permitted determination down to concentrations of 10 and 15.4 ng ml^{-1} (at a signal-to-noise ratio of 2) in aqueous solution and in serum, respectively. Fluorescence detection (excitation and emission wavelengths of 360 and 480 nm, respectively) exhibited somewhat poorer sensitivities for aqueous and serum samples: the respective limits of detection were 25 and 32 ng ml^{-1} at a signal-to-noise ratio of 2. Both the amperometric and the fluorescence detection were free from interference from blood components, but the fluorescence measurement required a post-column pH adjustment. UV photometric detection at 254 nm exhibited detection limits of 15 and 65 ng ml^{-1} in aqueous samples and in serum, respectively, and suffered from interferences from blood components that strongly absorbed in the ultraviolet region. All three detection techniques exhibited good linearity and precision.

INTRODUCTION

Narciclasine, (Fig. 1) originally obtained by extraction from narcissus bulbs [1], exhibits very interesting biological properties, such as antitumour acitivity [2,3], an antifeedant effect on the larvae of the butterfly *Eurema Hecabe Mandarina* and inhibition of bacterial growth and of the growth of wheat grain radicles [1]. Its antimitotic effect occurs during the metaphase through immediate termination of protein synthesis in eukaryotic cells at the peptide bond formation step, apparently because of an interaction with the anisomycin area of the ribosomal peptidyl transferase centre [2–6].

In a general study of narciclasine as an antitumour agent that is either directly

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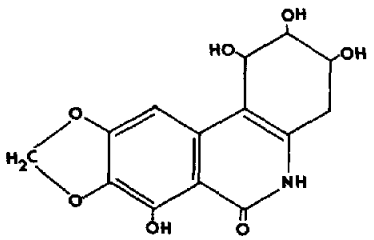

Fig. 1. Structure of narciclasine (1,2,3,7-tetrahydroxy-8,9-methylenedioxy-1,2,3,4-tetrahydrophenanthridone).

injected into a laboratory animal or released from polyHEMA matrix [7], a reliable method is required for determination of narciclasine in blood in order to evaluate its pharmacokinetics. So far, the substance has been determined by simple UV photometric and fluorescence methods [8] as it strongly absorbs radiation at *ca.* 254 nm and produces intense yellow fluorescence in alkaline solutions; however, there is no fluorescence below pH 12. These methods are not sufficiently selective for determination of narciclasine in biological samples. Therefore, a high-performance liquid chromatographic (HPLC) method has been developed and is described in this paper. In view of the character of narciclasine, a reversed-phase system was selected and optimized for analyses of blood samples. In addition to UV photometric and fluorescence detection, amperometric detection at a carbon electrode was studied, as narciclasine is electrochemically oxidizable.

EXPERIMENTAL

Apparatus

The chromatographic system consisted of a pump, either a JASCO 880-PU (Tokyo, Japan) or a Perkin-Elmer Series 3B (Norwalk, CT, U.S.A.), a Rheodyne injection valve (Cotati, CA, U.S.A.) with a 50-, 20- or 10-μl sampling loop, and a Separon SGX C_{18} glass column (7 μm particle size, 150 mm × 3.3. mm I.D.) from Tessek (Prague, Czechoslovakia). The detection system was a JASCO 875-UV intelligent UV–VIS detector, a Perkin-Elmer LS-4 fluorescence spectrometer or an ADLC 2 amperometric detector (Laboratorní Přístroje, Prague, Czechoslovakia) with a carbon fibre array working electrode, an Ag/AgCl reference electrode and a stainless-steel counter-electrode [9]. With fluorescence detection, the mobile phase pH was adjusted to a post-column value greater than 12 by pumping a 0.1 *M* sodium hydroxide solution with a JASCO 880-PU pump and mixing it with the mobile phase in a low-volume T-piece. The detector signals were recorded and processed using a Perkin-Elmer 561 or a Shimadzu C-R4A Chromatopac recorder (Düsseldorf, F.R.G.).

The measurements were carried out at laboratory temperature (20 ± 2°C), and the electrode potential values referred to a saturated aqueous Ag/AgCl electrode.

Chemicals

Narciclasine, isolated by the procedure described elsewhere [1], was kindly provided by Prof. G. Ceriotti of the Department of Pharmaceutical Science, Padova University, and was used as received. A stock solution (1 mg/ml) was prepared by dissolving the substance either in methanol or in water containing a small amount of acetic acid. The working solutions were prepared by appropriately diluting the stock solution with water immediately before measurement.

All the chemicals used were of analytical-reagent grade (Carlo Erba, Milan, Italy, and Merck, Darmstadt, F.R.G.). The mobile phase was degassed *in vacuo* and in an ultrasonic bath.

Sample preparation

The blood samples were obtained from mice of NCL strain with an average weight of 30 g. To evaluate the HPLC method, various amounts of narciclasine were added to the blood collected from the mice, proteins were removed and the supernatant liquid was analysed. The method was applied to analysis of the blood of mice to which 1 mg of narciclasine was administered peritoneally; the blood was collected after 15 min.

For protein precipitation [10], a 0.5-ml blood sample was mixed with 0.6 ml of a $CdSO_4$ solution (solution A) and 0.4 ml of a borate buffer of pH 11 (solution B). The mixture was centrifuged at 2500 *g* for 20 min. A 50-μl volume of the supernatant was then directly injected into the chromatographic system.

To make solution A, 10.2 g of $CdSO_4 \cdot 8H_2O$ were dissolved in 24 ml of 0.5 *M* H_2SO_4, and the solution was made up to 100 ml with water. To make solution B, 6.2 g of boric acid were dissolved in 150 ml of 1 *M* NaOH, and the solution was diluted to 1000 ml with water.

RESULTS AND DISCUSSION

In view of the chemical character of narciclasine and the blood samples to be analysed, a reversed-phase chromatographic system was selected with a C_{18} stationary phase and a mobile phase of water and methanol. Potassium dihydrogenphosphate was added so that the pH of the mobile phase could be maintained within an optimal range, and also to attain a sufficient electrical conductivity of the mobile phase for amperometric detection. The experimental conditions were then optimized with respect to the narciclasine capacity ratio and to the sensitivity of amperometric detection. The conditions for UV photometric and fluorescence detection had been established from previous direct determinations of narciclasine [8].

As could be expected, the capacity ratio of narciclasine decreases with increasing methanol content in the mobile phase (Fig. 2). The sensitivity of amperometric detection strongly depends on the methanol content (Fig. 2): the response increases with increasing concentration of methanol, probably owing to an in-

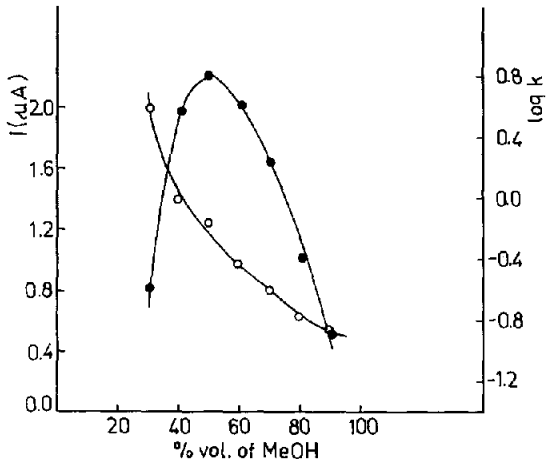

Fig. 2. Dependence of the capacity ratio (○) and of the amperometric signal (●) for narciclasine on the methanol content in the mobile phase. Mobile phase, methanol–0.025 *M* KH_2PO_4 (pH 5.5), 50:50 (v/v); flow-rate, 0.3 ml min^{-1}; potential, +1.8 V.

creased solubility of narciclasine in the mobile phase, attains a maximum at a methanol-to-water ratio of *ca.* 50:50 (v/v) and then decreases again, apparently owing to adsorption of methanol on the electrode surface causing its partial passivation. It follows that the 50% methanol concentration is optimal both for the narciclasine retention and its amperometric detection.

A hydrodynamic voltammogram of narciclasine, obtained with the mobile phase containing 50% (v/v) methanol (Fig. 3), confirms that the optimal potential of the working electrode is +1.8 V.

Fig. 4 indicates that neither the narciclasine capacity ratio nor the amperometric signal is strongly affected by the mobile phase pH within the range acces-

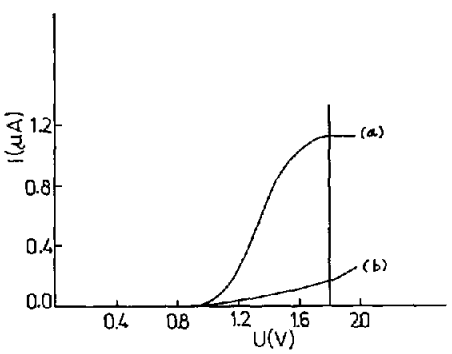

Fig. 3. Hydrodynamic voltammogram of narciclasine (a) and the background current (b). Mobile phase, methanol–0.025 *M* KH_2PO_4 (pH 5.5), 50:50 (v/v); flow-rate, 0.3 ml min^{-1}.

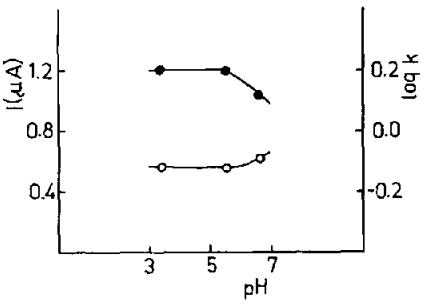

Fig. 4. Dependence of the capacity ratio (○) and of the sensitivity of amperometric detection (●) on the mobile phase pH. Mobile phase, methanol–0.025 *M* KH_2PO_4, 50:50 (v/v), pH adjusted by addition of sodium hydroxide and orthophosphoric acid solutions; flow-rate, 0.3 ml min^{-1}.

sible with C_{18} stationary phases. This is to be expected, because the acid–base properties of narciclasine are not pronounced. Therefore, pH 5.5 is optimal for practical work.

The dependences on the salt concentration in the mobile phase (Fig. 5) demonstrate that the sensitivity of the amperometric detection increases somewhat with increasing content of the salt, owing to increasing electrical conductivity of the mobile phase, whereas the capacity ratio decreases, as the mobile phase polarity is decreasing with increasing ionic strength.

Hence the optimal mobile phase is methanol–aqueous 0.025 *M* potassium dihydrogenphosphate (50:50, v/v), pH 5.5. The mobile phase flow-rate was maintained at a value of 0.3 ml min^{-1}, which led to a convenient retention time of narciclasine of *ca.* 5 min. Under these conditions, the optimal electrode potential was +1.8 V. As reported previously [8], UV photometry is conveniently carried out at 254 nm, whereas the fluorescence measurement requires an excitation wavelength of 360 nm and measurement of the emitted radiation at 480 nm. Of

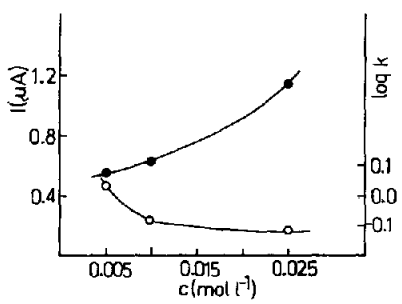

Fig. 5. Dependence of the capacity ratio (○) and of the sensitivity of amperometric detection (●) on the salt concentration in the mobile phase. Mobile phase, methanol–0.025 *M* KH_2PO_4, 50:50 (v/v); flow-rate, 0.3 ml min^{-1}.

TABLE I

DETERMINATION OF NARCICLASINE IN AQUEOUS SOLUTIONS AND SERUM

Detection method	Sample	Limit of detection[a] (ng ml^{-1})	Linear dynamic range (μg ml^{-1})	Correlation coefficient	Precision (R.S.D.) (%)		
					0.1 μg ml^{-1}	0.5 μl ml^{-1}	1.0 μg ml^{-1}
Voltammetric	Aqueous	10	0.01–10	0.9936	6.5	3.2	1.4
(+1.8 V)	Serum	15	0.015–10	0.9991	7.2	4.5	1.9
UV photometric	Aqueous	15	0.015–12	0.9999	8.5	2.8	1.2
(λ = 254 nm)	Serum	65	0.065–10	0.9944	10.4	3.5	1.6
Fluorimetric	Aqueous	25	0.015–5	0.9986	5.8	2.5	1.0
($\lambda_{exc.}$ = 360 nm; $\lambda_{em.}$ = 480 nm)	Serum	32	0.032–5	0.9984	6.1	3.2	1.5

[a] At a signal-to-noise ratio of 2.

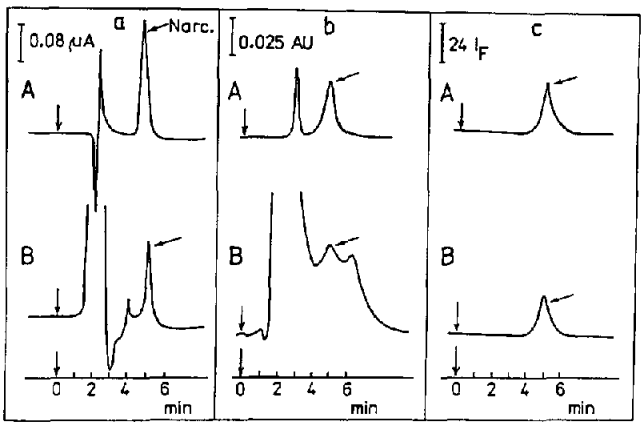

Fig. 6. Chromatogram of aqueous solutions (A) and blood samples (B) containing 0.5 μg of narciclasine in 1 ml with amperometric (a), UV photometric (b) and fluorimetric (c) detection. Mobile phase, methanol–0.025 *M* KH_2PO_4, 50:50 (v/v), pH 5.5; flow-rate, 0.3 ml min^{-1}.

course, a drawback of fluorescence detection is the neccssity for the post-column pH to be adjusted to a value greater than 12 (see Experimental).

Chromatograms of narciclasine standards in aqueous solutions and in blood samples pretreated as described in Experimental are depicted in Fig. 6, and the results obtained with the three detection methods are summarized in Table I. It can be seen that the amperometric technique is more sensitive than the other two methods. Moreover, the post-column pH adjustment involved in the fluorescence detection causes the narciclasine peak to broaden a little. The apparatus is also more complex, and pumping of a strongly alkaline corrosive solution is required. UV photometry is least sensitive in blood analysis and suffers from interference from blood components, whereas amperometry and fluorimetry do not.

The other analytical paramcters are comparable for the three detection methods and are satisfactory (Table I). There is no significant difference in the analytical parameters obtained from measurement of the peak heights and areas: all the data reported here are based on the peak-area measurement. The absolute calibration method was employed for quantitative analysis, as we were unable to find an internal standard that would produce a sufficiently intense amperometric or fluorescence signal.

The relative standard deviations obtained with the three detection methods are similar. Typical values (four parallel determinations) are given in Table I.

The method, with amperometric and fluorescence detection, was then applied to the determination of narciclasine in the blood of mice, 15 min after peritoneal administration of 1 mg of narciclasine. Three parallel determinations were carried out, using the absolute calibration method. The results from amperometric and fluorescence measurements are in excellent agreement: 0.716 ± 0.069 and 0.713 ± 0.098 μg ml^{-1}, respectively. Thus it can be seen that, for mice with a

blood total volume of *ca.* 3 ml, the amount of narciclasine contained in the blood after 15 min was 0.25% of the amount administered.

Therefore, the method as developed is readily applicable to practical analyses of this type, with amperometric detection preferred as it is most sensitive and simplest. The method is being used to monitor narciclasine concentrations in the blood of mice during pharmacokinetic studies, and the results will be published elsewhere.

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