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DETERMINATION OF FLUORESCENT DIAMIDINES IN PLASMA OF EXPERIMENTAL ANIMALS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The fluorescent diamidines (*E*)-2,2′-vinylenedi-1-benzo[*b*]furane-5-carboxamidine dihydrochloride (I) and 2-[2-(6-amidinoindole-2-yl)-(*E*)-vinyl]-1-benzofurane-5-carboxamidine dihydrochloride (II) were determined in the plasma of experimental animals by high-performance liquid chromatography with a mobile phase of methanol–water (60:40, v/v) containing 0.005 *M* octanesulphonic acid and 0.003 *M* dimethyloctylamine. Samples were prepared by precipitation of plasma proteins with methanol–perchloric acid. Quantitation was performed by measuring the peak heights after monitoring the native fluorescence. The assay was linear over the range 5–750 ng/ml for I and 5–500 ng/ml for II, with limits of determination of 2.5 ng/ml for I and 1.5 ng/ml for II. Coefficients of variation were below 10% at all concentrations studied.

INTRODUCTION

Pharmacological investigations of heterocyclic diamidines have shown that these compounds exhibit a wide variety of therapeutically interesting activities in experimental animals as well as in humans. After the success of stilbamidine and later pentamidine in the treatment of human trypanosomiases, followed by diminazene for the treatment of bovine trypanosomiases [1], Dann and co-workers [2,3] synthesized heterocyclic diamidines, which have shown activity both in human and bovine infections [4,5]. Some diamidines also showed antibacterial and antifungal [6] or antiviral [7] activities. Recent results [8] have focused on the activity of diamidines consisting of two heterocyclic ring systems linked

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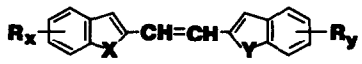

R_x	at	X	R_y	at	Y	
Am	5	O	Am	5	O	I
Am	5	O	Am	6	NH	II

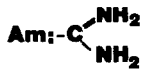

Fig. 1. Chemical structures of the antileukemic diamidines (*E*)-2,2′-vinylenedi-1-benzo[*b*]furane-5-carboxamidine dihydrochloride (I) and 2-[2-(6-amidinoindole-2-yl)-(*E*)-vinyl]-1-benzofurane-5-carboxamidine dihydrochloride (II) of type C.

by an ethylene bridge (i.e. the so-called type C [7], see Fig. 1) against transplanted leukemia in mice.

However, until now no reliable data have been reported on the pharmacokinetic properties of any of these compounds. Data already published rely entirely on the unspecific methods of measuring UV absorption [9,10] or fluorescence [11,12] — either native or after derivatization — in relatively crude extracts. Recently, methods have been reported for the determination of diminazene by gas chromatography–mass spectrometry [13] or by high-performance liquid chromatography (HPLC) [14,15]. However, a more thorough investigation, which is also applicable to other compounds of this class, is still lacking.

Recently we have published a method for the determination of similar compounds using quantitative thin-layer chromatography (TLC) [16]. In the present paper we describe a quantitative method for the assessment of several diamidines by HPLC. This method has been fully elaborated for compounds (*E*)-2,2′-vinylenedi-1-benzo[*b*]furane-5-carboxamidine dihydrochloride (I) and 2-[2-(6-amidinoindole-2-yl)-(*E*)-vinyl]-1-benzofurane-5-carboxamidine dihydrochloride (II) (for chemical formulae see Fig. 1), and has already proved its reliability and accuracy in pharmacokinetic studies in experimental animals.

EXPERIMENTAL

Materials

Octanesulphonic acid was obtained from Serva (Heidelberg, F.R.G.). All other reagents were analytical grade and obtained from Merck (Darmstadt, F.R.G.). Dimethyloctylamine (DMOA) was synthesized as described previously [18]. Compounds I and II were synthesized at the Institut für Pharmazie und Lebensmittelchemie, University of Erlangen-Nürnberg, according to methods described previously [3,8].

Apparatus

The HPLC apparatus consisted of an A 110 pump and an SFM 23 fluorimeter (Kontron, Eching, F.R.G.), a strip-chart recorder (LKB, Gräfelfing, F.R.G.) and an automated integrator (Minigrator, Spectra-Physics, Darmstadt, F.R.G.).

Samples were injected using a Rheodyne valve (Type 7125, Rheodyne, Contato, CA, U.S.A.) with a 100-μl loop. The loop was filled with a plastic disposable syringe (Terumo, Haasrode, Belgium) connected to a 0.22-gauge needle with a flat tip. Columns (250×4.6 mm I.D., Bischoff, Leonberg, F.R.G.) were equipped with stainless-steel frits and packed in the laboratory with Hypersil 5-μm ODS (Shandon, Runcorn, U.K., obtained from Bischoff).

Sample preparation

Stock solutions of the compounds of interest were prepared by dissolving appropriate amounts of compounds I and II in water to give final concentrations of 40 μg/ml. These stock solutions were further diluted with bovine citrate plasma to give final concentrations of 500, 300, 200, 100, 75, 50, 25, 10 and 5 ng/ml. All concentrations refer to the concentration of the free base, irrespective of the diamidine salts used.

For sample preparation, 200 μl of these standards or of the unknowns were placed into polypropylene microreaction vessels (Sarstedt, Nümbrecht, F.R.G.), 100 μl of methanol, cooled to 4–8°C, were added, and the resulting mixture was shaken under protection from light for 15 min. Then 100 μl of a cooled mixture of methanol–70% perchloric acid (5:1, v/v) were added, thoroughly mixed and centrifuged at 4°C and ca. 25 000 *g*. The resulting supernatant, at least 125 μl, was injected into the liquid chromatographic system.

Chromatography

Compounds I and II were chromatographed on Hypersil 5-μm ODS with a mobile phase of methanol–water (60:40, v/v) containing 0.005 *M* octanesulphonic acid and 0.003 *M* DMOA. The pH of this mixture was adjusted to 3.0.

Chromatography was performed at room temperature (ca. 22°C) at a flow-rate of 1.0 ml/min. By use of an injection valve, exactly 100 μl of the sample were injected. Excitation and emission wavelengths are given in Table I.

Quantitation

Quantitation was performed using an external standard method. Peak heights of the unknowns were compared with a calibration curve constructed by adding the amounts of compounds I and II given above to blank plasma samples.

TABLE I

EXCITATION AND EMISSION WAVELENGTHS FOR QUANTITATION OF COMPOUNDS I AND II

Compound	Excitation wavelength (nm)	Emission wavelength (nm)
I	377	407
II	394	456

Recovery

Recovery was determined by taking spiked plasma samples of different origins through the extraction procedure outlined above and comparing the peak heights with those of samples diluted in water and treated likewise.

Precision and accuracy

The precision and accuracy of the assay were determined by adding known amounts of the compounds of interest to blank plasma samples. Multiple samples were analysed as described above, and the results obtained were compared with the peak heights obtained by injecting corresponding amounts of the compounds of interest without further treatment.

RESULTS AND DISCUSSION

Methods hitherto published for other diamidines have been found unsuitable for the determination of compounds I and II. We recently reported a method for the determination of trypanocidal diamidines [16] using quantitative TLC. However, this method requires extensive sample preparation and a time-consuming chromatographic method. Therefore we developed an alternative method using HPLC for the determination of compounds I and II.

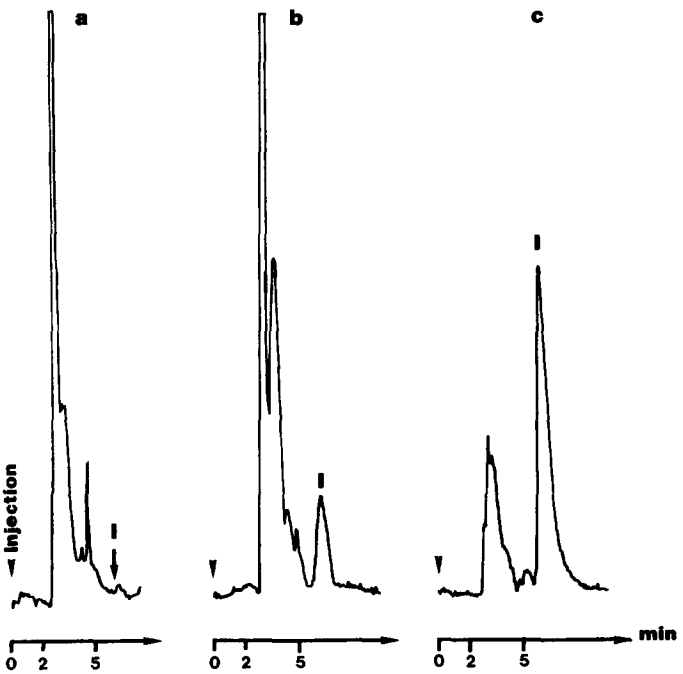

Fig. 2. Chromatograms of (a) compound I from a blank plasma sample, (b) a standard plasma sample containing 25 ng/ml and (c) a sample of mouse plasma containing 101 ng/ml. Settings of the fluorescence detector were: excitation wavelength, 377 nm; emission wavelength, 407 nm; sensitivity, HIGH 10.0.

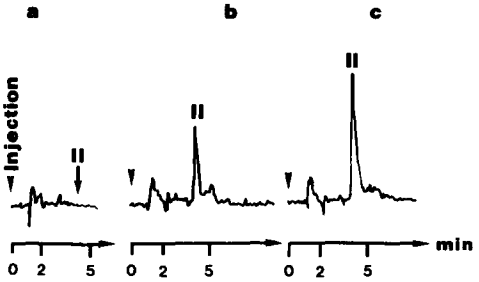

Fig. 3. Chromatograms of compound II from (a) a blank plasma sample, (b) a sample of mouse plasma containing 17 ng/ml, and (c) a standard plasma sample containing 25 ng/ml. Settings of the fluorescence detector were: excitation wavelength, 394 nm; emission wavelength, 456 nm; sensitivity, HIGH 10.0

Chromatograms of these compounds obtained by the described method are shown in Figs. 2 and 3. Both compounds are well separated from possibly interfering endogenous material. Since amidines are very prone to adsorption, even on reversed-phase packings, owing to their high pK_a value of ca. 11 [18], DMOA was added to the mobile phase as a displacer. On several brands of reversed-phase packings we observed a severe reduction or even loss of peak heights of compounds I and II. Furthermore, severe tailing was observed on some other materials. This latter fact has already been observed by others [19,20] and could be overcome by addition of DMOA to the mobile phase. Therefore a careful choice should be made when selecting the proper stationary phase for the determination of similar strongly basic compounds.

Likewise, amidines are strongly adsorbed on the surface of laboratory glassware [16]. Therefore care was taken to perform sample preparation only in plastic disposables. In particular, the glass syringes commonly used for HPLC injection had to be replaced by plastic ones.

The most suitable method of sample preparation was shaking the sample with methanol to dissociate protein binding [21], followed by precipitation of plasma proteins by methanol–70% perchloric acid. Recoveries from plasma samples of different origins were almost complete, as shown in Table II.

TABLE II

RECOVERY OF COMPOUNDS I AND II FROM PLASMA SAMPLES

Concentration (ng/ml)	Recovery (%)	
	I	II
1000	81	–
500	88	–
100	100	98
25	103	102
10	100	104
5	98	99

TABLE III

LINEARITY OF THE ASSAY OF COMPOUNDS I AND II AT DIFFERENT CONCENTRATIONS AND SETTINGS OF THE DETECTOR

Range of concentrations and setting	Compound I				Compound II			
	a (mean ± S.D.)	*b* (mean ± S.D.)	r^2	*n*	*a* (mean ± S.D.)	*b* (mean ± S.D.)	r^2	*n*
1–0.5 μg/ml (MED 10.0)	0.1107 ± 0.18	0.0298 ± 0.009	0.9974 ± 0.002	5	1.4412 ± 0.464	0.0187 ± 0.003	0.9717 ± 0.022	5
25–100 ng/ml (HIGH 10.0)	0.0288 ± 0.117	0.1351 ± 0.019	0.9399 ± 0.072	10	−0.1469 ± 0.412	0.1127 ± 0.017	0.9869 ± 0.012	5
0–10 ng/ml (HV VAR 10.0)	0.120 ± 0.074	1.664 ± 0.268	0.9992 ± 0.001	5	−0.0864 ± 0.5429	1.0488 ± 0.133	0.9763 ± 0.022	5

Quantitation was performed using an external standard procedure. Calibration curves had to be divided into three concentration ranges since one setting of the detector did not cover the range of all possible concentrations. For actual samples used in pharmacokinetic studies, these settings were chosen according to the concentrations expected from preliminary experiments. The linearity of the assay for both compounds I and II at different settings is given in Table III. These data were acquired over a period of two months for all sets of data.

TABLE IV

PRECISION AND ACCURACY OF THE ASSAY (INTER-DAY VARIATIONS) FOR COMPOUNDS I AND II

Concentration added (ng/ml)	Compound I				Compound II			
	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Error (%)	*n*	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Error (%)	*n*
5	5.0 ± 0.42	8.5	−1.3	8	5.1 ± 0.34	6.6	+2.8	5
10	10.0 ± 0.75	7.5	−0.4	7	9.6 ± 0.92	9.6	−3.7	5
25	26.7 ± 2.23	8.3	+6.8	9	27.7 ± 2.64	9.6	+9.7	5
50	52.6 ± 4.46	8.5	+5.1	11	48.8 ± 2.44	5.0	−2.4	5
75	74.8 ± 5.86	7.8	−0.2	8	80.1 ± 3.76	4.7	+6.8	4
100	97.7 ± 4.78	4.9	−2.3	11	107.6 ± 4.49	4.2	+7.6	4
250					268.0 ± 20.9	7.8	+7.2	5
300	292.0 ± 8.47	2.9	−2.6	4				
500	514.2 ± 9.77	1.9	+2.8	5	500.8 ± 27.04	5.4	+0.2	4

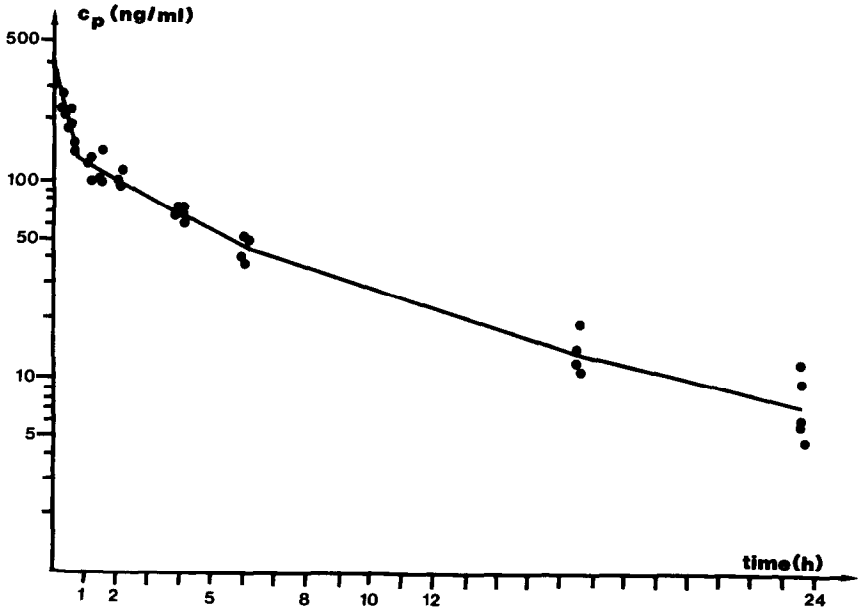

Fig. 4. Plasma levels of compound I in mice after administration of 10 mg/kg intraperitoneally. Each data point was obtained from one mouse.

The precision of the assay is summarized in Table IV for compounds I and II. In each case coefficients of variation are well below 10%. The accuracy of the assay is also given in Table IV. Deviations from the amounts added to blank samples are below 10% in all cases.

The assay procedure described has already proved its reliability and accuracy in studies in experimental animals [22]. A plasma concentration–time curve for a typical set of experiments is shown in Fig. 4. Moreover, this method is more specific than the presently available methods of measuring total fluorescence and less time-consuming than our previously reported TLC-method [16].

From the data presented we therefore conclude that this assay is a very sensitive, accurate and reliable method for the determination of the antileukemic and fluorescent diamidines of type C in the plasma of experimental animals, which can be used for pharmacokinetic studies and also for drug monitoring.

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