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DETERMINATION OF FLUORESCENT TRYPANOCIDAL DIAMIDINES BY QUANTITATIVE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The fluorescent trypanocidal diamidines 2-(4-amidinophenyl)indole-6-carboxamidine dihydrochloride (I, DAPI), 2-(4-amidinophenyl)benzo[*b*]thiophene-6-carboxamidine dihydrochloride (II) and 2-(4-amidinophenyl)-1-benzofurane-5-carboxamidine dihydrochloride (III) were determined in plasma, urine, faeces and tissues of experimental animals using quantitative thin-layer chromatography. Samples were extracted with *n*-octanol after addition of sodium hydroxide and subsequently re-extracted into 0.1 *M* hydrochloric acid. Chromatography was performed on silica gel plates under nitrogen with *n*-butanol saturated with 2 *M* hydrochloric acid. Quantitation was performed by measuring native fluorescence using a fluorodensitometer. The respective diamidines were used as internal standards for each other to ensure precision (coefficient of variation < 7%) and accuracy of the assay. Calibration curves were linear up to 150 ng/ml of sample solution with detection limits of 10 ng/ml of sample solution for I and III and 50 ng/ml for II. The described method has been successfully used for pharmacokinetic studies in experimental animals.

INTRODUCTION

Since 1939, stilbamidine and later pentamidine have played an important role in the treatment of human trypanosomiasis [1], whereas a diamidine compound effective against bovine infections was only developed considerably later, being introduced as diminazene in 1955 [2]. However, its unstable chemical structure limits its use for prophylaxis. Therefore Dann et al. [4]

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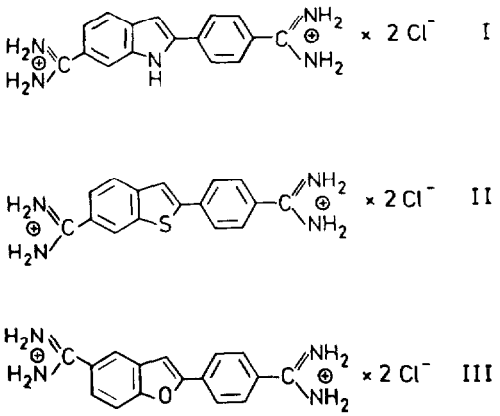

Fig. 1. Formulae of compounds I (DAPI), II and III.

synthesized the fluorescent, stable diamidines III [2-(4-amidinophenyl)-1-benzofurane-5-carboxamidine dihydrochloride] and I [2-(4-amidinophenyl)-indole-6-carboxamidine dihydrochloride, DAPI], being active like diminazene in animals [5], and II [2-(4-amidinophenyl)benzo[*b*]thiophene-6-carboxamidine dihydrochloride] [3], being active in human beings [6] (for formulae, see Fig. 1).

Owing to the lack of suitable analytical methods, until now no reliable data on the pharmacokinetics of any of these diamidines have been reported. Data hitherto published rely on measurement of UV absorption [7, 8] or of native fluorescence [9–12]. Derivatization [13, 14] considerably improved sensitivity; however, the detection limit in body fluids is still above those levels obtained after administration of therapeutic doses. Measurement of total radioactivity after administration of ^{14}C-labelled compounds [15, 16] is very sensitive, but does not discriminate among possible metabolites. Paper chromatographic methods [14, 16] are not suitable for quantitative determinations. Methods using high-performance liquid chromatography (HPLC) [17, 19] and gas chromatography—mass spectrometry (GC—MS) [18] have been reported recently for diminazene; the HPLC methods are not sensitive enough for our compounds and the GC—MS method requires costly instrumentation and is only applicable for diminazene.

Therefore, in the present paper, a simple and effective thin-layer chromatographic (TLC) method is proposed for pharmacokinetic studies with compounds I, II and III in experimental animals.

EXPERIMENTAL

Materials

Compounds I, II and III were synthesized at the Institut für Pharmazie, University of Erlangen-Nürnberg (Erlangen, F.R.G.), according to methods described previously [3, 4] and were used as their hydrochloride salts.

All solvents and reagents used were of analytical grade (Merck, Darmstadt, F.R.G.) and checked for possible interfering fluorescent impurities.

TLC plates were prepared by coating glass plates (20 × 20 cm) with 0.2 mm

of silica (60 HR, Merck) according to the specifications of the manufacturer using a commercially available apparatus (UNOPLAN, Shandon, Runcorn, U.K.). Before use, the TLC plates were activated by heating to 80°C for 1 h and then to 110°C for another hour. Special care was taken to protect them from dust and other possible fluorescent laboratory contamination.

Sample preparation

Sample preparation as outlined in Fig. 2 was carried out with the volumes of solutions given in Table I. These volumes had to be adjusted according to the concentrations expected from previous experiments. The respective amounts of the internal standard and the compounds of interest had to be within the same range, but below 150 ng per spot to ensure a linear calibration curve.

Plasma and urine samples were only diluted, whereas faeces and tissues were homogenized together with the internal standard solution and a 0.9% solution of sodium chloride. Homogenates were left at ambient temperature for 3 h to ensure adequate binding of the internal standard. The apparatus for homogenization (Bühler, Tübingen, F.R.G.) was equipped with polypropylene vessels (Sarstedt, Nümbrecht, F.R.G.) to minimize adsorption. All concentrations reported refer to the respective base content of the compounds to be determined. Solutions of these compounds and of their respective internal standards were prepared to a final concentration of 10 μg/ml. Standard solutions were further diluted to give the concentrations necessary for construction of a calibration curve.

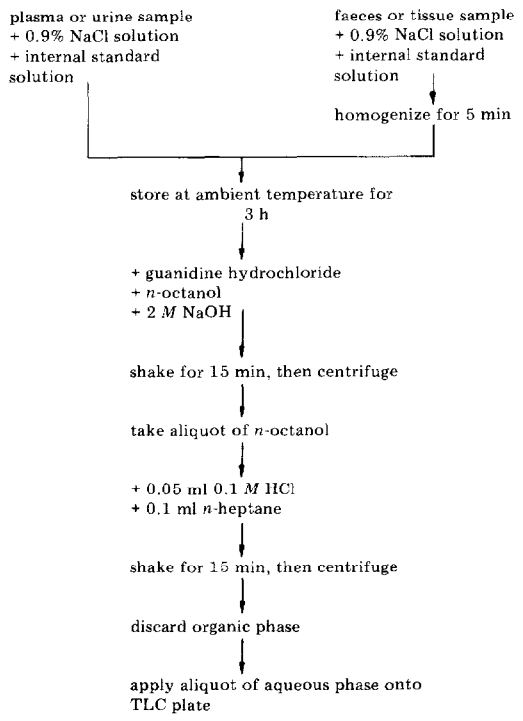

Fig. 2. Schematic outline of sample preparation.

TABLE I

VOLUMES USED IN THE PREPARATION OF PLASMA, URINE, FAECES AND TISSUE SAMPLES

	Plasma/urine	Faeces	Tissues
Sample	1.0 ml	3.0 g	2.0 g
0.9% Sodium chloride solution	1.0 ml	30.0 ml	20.0 ml
Internal standard	0.02—0.05 ml	0.05—1.0 ml	0.05—1.0 ml
Guanidine hydrochloride	0.1 g	1.5 g	1.0 g
n-Octanol	1.0 ml	15 ml	10 ml
2 *M* Sodium hydroxide	0.5 ml	7.5 ml	5.0 ml
0.1 *M* Hydrochloric acid		0.05 ml	
Octanol phase from extraction		0.05—0.5 ml	
Octanol		up to 0.5 ml	
n-Heptane		0.1 ml	
Applied to TLC plate		0.005—0.02 ml	

Chromatography

Aliquots of 5—20 μl of the final aqueous sample solution were applied to the TLC plate using a PTFE-tipped microlitre syringe (Macherey & Nagel, Düren, F.R.G.). Standards were prepared by applying several solutions with different concentrations of the compound of interest, but the same concentration of the internal standard.

TLC plates were chromatographed under a nitrogen atmosphere with a solvent consisting of *n*-butanol saturated with 2 *M* hydrochloric acid. Chromatography was usually performed overnight. The resulting chromatograms were dried at 50° C under vacuum for about 30 min.

Measurement

Quantitative evaluation of the spots was performed using a fluorodensitometer (KM 3, Zeiss, Oberkochen, F.R.G.) connected to an automated integrator (Minigrator, Spectra-Physics, Darmstadt, F.R.G.) in the sample-monochromator (Pr-M) setting. The appropriate excitation wavelength of an St 41 mercury medium-pressure lamp was selected by a band filter, whereas the emission wavelength was selected by the monochromator. The respective values were determined by scanning the emission spectra in situ at the excitation wavelengths of 313 and 365 nm, respectively [20]; the resulting values are given in Table II.

TABLE II

INTERNAL STANDARDS AND EMISSION AND EXCITATION WAVELENGTHS FOR THE DETERMINATION OF COMPOUNDS I, II AND III

Compound	Internal standard	Excitation wavelength (nm)	Emission wavelength (nm)
I	III	365	450
II	I	313	405
III	I	313	408

TABLE III

VALUES OF Q (RATIO OF RECOVERIES OF INTERNAL STANDARD/COMPOUND OF INTEREST) FOR COMPOUNDS I, II AND III FROM DIFFERENT SAMPLES

Sample	Concentration range (μg/g or μg/ml)	Q value		
		I	II	III
Plasma	0.02— 0.2	1.15	1.0	0.9
Urine	0.02— 0.2	1.45	0.6	0.7
Faeces	0.2 — 2.0	2.15	0.55	0.46
Tissues	1.0 —10.0	1.35	0.85	0.74

Calculations

Amounts of the compound of interest on the plate were calculated from a regression line of standard amounts versus peak areas.

These measured amounts were standardized to an applied volume of 10 μl (c_m) and then corrected to give the true concentrations per millilitre (c_{true}) for plasma and urine, using the formula

$$c_{true} = \frac{0.5\, c_m}{R_{I.S.}\, V}\, Q$$

where $R_{I.S.}$ is the recovery of the internal standard, V is the volume of *n*-octanol used in the last extraction step and Q is the ratio of recoveries between internal standard and the compound of interest, as given in Table III.

For faeces and tissues, the following equation was used:

$$c_{true} = \frac{c_m\, V_{oct}}{R_{I.S.}\, V\, 2G}\, Q$$

where V_{oct} is the volume of *n*-octanol used in the first extraction step and G is the weight of the sample.

The respective values for Q in Table III were determined by spiking blank samples with different amounts of the compounds of interest and taking these through the extraction procedure as outlined above.

Recovery

Recovery was determined by adding known amounts of compounds I, II and III to blank plasma, urine, faeces and tissue samples and leaving them at ambient temperature for 3 h before taking them through the extraction procedure; peak areas were compared to those obtained from standard solutions.

Adsorption to different materials

Adsorption to glass was determined using 0.05, 0.1, 0.2, 0.5, 1.0, 5.0 and 10.0 μg/ml solutions of the respective compounds. The amount recovered from a solution after adding 10% guanidine hydrochloride was considered to be complete, since raising guanidine concentrations did not further improve recovery.

TABLE IV

RECOVERY OF I, II AND III FROM PLASMA, URINE, FAECES AND TISSUE SAMPLES AT DIFFERENT CONCENTRATIONS

Sample	Concentration (μg/ml or μg/g)	Recovery (mean ± S.D.) (%)		
		I	II	III
Plasma	0.05	42.5 ± 9.6	54.9 ± 6.8	95.8 ± 10.9
	0.10	60.7 ± 6.6	57.0 ± 3.0	71.1 ± 6.2
	0.20	68.0 ± 5.6	57.0 ± 7.4	82.2 ± 7.3
Urine	0.05	57.5 ± 11.5		88.7 ± 6.8
	0.10	56.0 ± 14.5	69.2 ± 3.3	82.0 ± 4.2
	0.25	62.0 ± 7.7	56.4 ± 7.8	82.4 ± 5.8
	0.50	52.9 ± 11.4	61.8 ± 11.9	
	1.00		52.9 ± 3.8	82.6 ± 4.0
Faeces	0.5	19.0 ± 3.5		46.7 ± 6.4
	1.0	26.6 ± 5.6		53.2 ± 12.9
	2.0	15.8 ± 3.3	32.4 ± 3.7	
	5.0	14.4 ± 3.3	25.1 ± 1.4	
	10.0	10.6 ± 1.0		31.1 ± 1.0
Tissue	1.0	58.4 ± 7.9		
	2.5	46.4 ± 1.3		
	5.0		58.1 ± 2.7	
	25.0	77.0 ± 4.2		112.0 ± 8.2
	40.0		85.5 ± 9.7	
	50.0	69.4 ± 9.8		103.0 ± 7.4

TABLE V

LOSS OF I, II AND III OWING TO ADSORPTION TO DIFFERENT MATERIALS FROM A 1 μg/ml SOLUTION

Recovery from polypropylene tubes was considered to be 100%.

Compound	Loss (%) for		
	Glass	Cellulose nitrate	Polypropylene
I	17	22	0
II	40	11	0
III	8	5	0

Loss due to adsorption to different materials was determined by storing 1.0 μg/ml solutions of the compounds for 15 h in vessels of the materials given in Table V; recovery from polypropylene tubes was considered to be complete since addition of 10% guanidine hydrochloride did not increase peak areas.

Loss of fluorescence by irradiation

Aliquots of 50 ng per spot of compounds I, II and III were applied to the TLC plates. Fluorescence intensity of these spots was measured versus time under continuous irradiation (Fig. 6). Fluorescence intensity was monitored continuously on an attached strip-chart recorder for the time indicated.

RESULTS AND DISCUSSION

Methods hitherto described have been found unsuitable for the determination of compounds I, II and III in experimental animals after therapeutic doses. From a large number of TLC solvents evaluated, only the one described resulted in a separation sufficient enough for the use of diamidines as internal standards for each other. Since diamidines show an extraordinarily high affinity to the surfaces of laboratory equipment, as well as to anionic macromolecules like nucleic acids and proteins, their use as an internal standard for each other was essential to obtain reliable results.

Chromatograms obtained with the described method are shown in Figs. 3 and 4. Evidently, the compounds of interest and their respective internal standards are well separated from each other. No interference was observed from endogenous fluorescent compounds, nor did possible metabolites [23] interfere with the assay.

Adsorption of basic compounds to laboratory glassware is a well known fact [21] and amidines are especially prone to this due to their high pK_a values of approximately 11 [22]. Displacement of bases by addition of alcohols [21] as well as competing basic compounds has been described and, therefore, an addition of guanidine hydrochloride was chosen to improve recovery. Addition of more than 10% did not further increase recovery; therefore, this was considered sufficient for our purposes. Adsorption was most marked at the low concentrations that are encountered in experimental animals following therapeutic doses, increasing steeply below 1.0 μg/ml. However, even at the highest concentration of 10 μg/ml, recovery in glass vessels was only 70, 78 and 100% for compounds I, II and III, respectively (Fig. 5). This effect is less obvious in plastic, especially polypropylene vessels (see Table V) and therefore caution was taken to execute most steps of the sample preparation in this material.

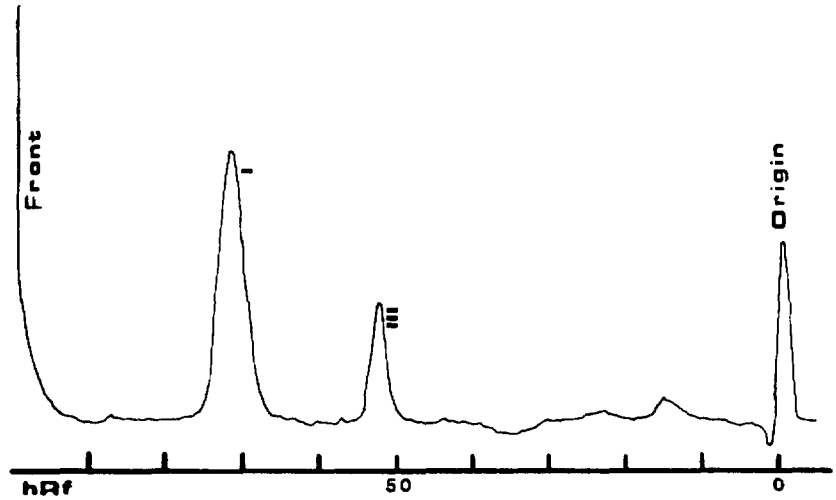

Fig. 3. Fluorescence intensity curve of a chromatogram of 1 ml of plasma containing 50 ng/ml I and 25 ng of the internal standard III (excitation wavelength 365 nm, emission wavelength 450 nm).

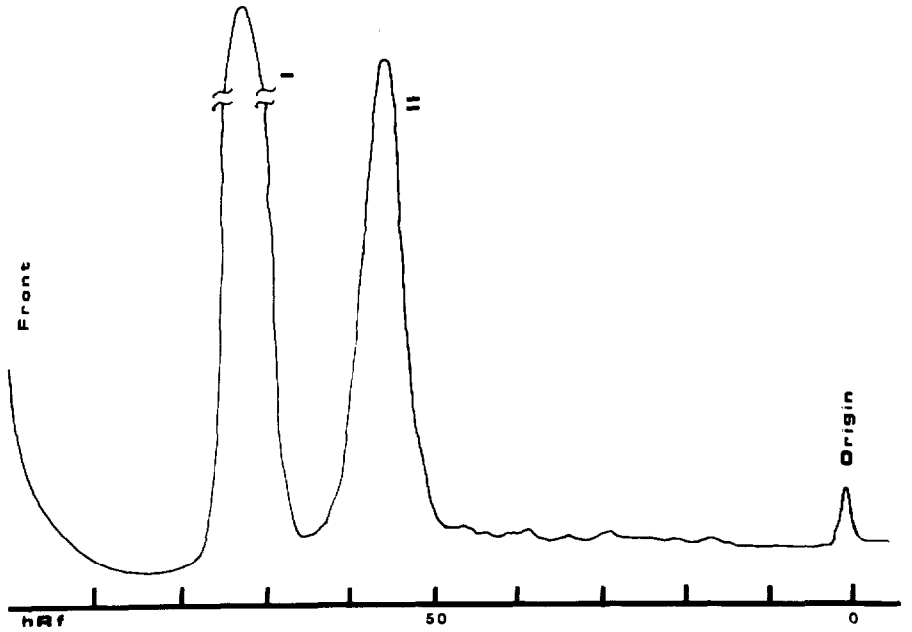

Fig. 4. Fluorescence intensity curve of a chromatogram of 1 ml of plasma containing 25 ng/ml II and 25 ng of the internal standard I (excitation wavelength 313 nm, emission wavelength 405 nm).

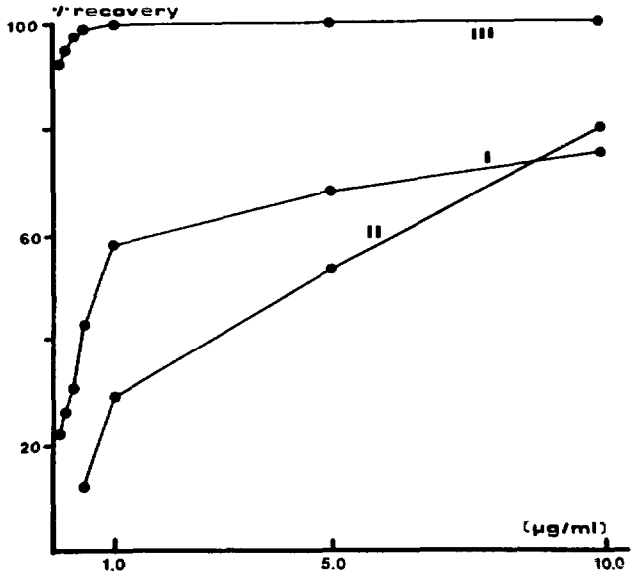

Fig. 5. Influence of the concentration of compounds I, II and III on their adsorption to glass reaction vessels from an aqueous solution.

Neither during the extraction procedure nor during storage of frozen samples did the compounds tested show any decomposition to known degradation products.

TABLE VI

PLATE-TO-PLATE VARIATION OF THE ASSAY AFTER EXTRACTION OF PLASMA SAMPLES

Settings of the amplifier were different for each concentration to give peak areas within the same range.

Compound	Amount applied (ng per spot)	Peak area (mV · s)	Coefficient of variation (%)
I	10	2100	2.8
	50	1800	1.9
II	10	1020	6.5
III	10	1540	3.1

Recovery of the compounds investigated is given in Table IV for different samples and concentrations. Though it was quite different for each diamidine, depending on the nature of samples and concentrations, the ratio of the respective recoveries of the compound of interest and the internal standard was quite constant, allowing for a common correction factor. Values for the ratio of recoveries are given in Table III.

Calibration curves were linear over the ranges 10—150 ng per spot for compounds I and III and 50—200 ng per spot for compound II. As an example, the calibration curve for compound I is shown in Fig. 7.

Precision of the method was determined by multiple analysis of the same spiked control plasma. The results given in Table VI show that for amounts of 10—150 ng per spot, coefficients of variation were well below 10%. Care was taken to modify the extraction procedure so that amounts applied to the TLC plate were always within this range.

Except for the less fluorescent compound II, the lower limits of detection were 10 ng/ml in plasma and 50—100 ng/ml (or ng/g) in urine, faeces and

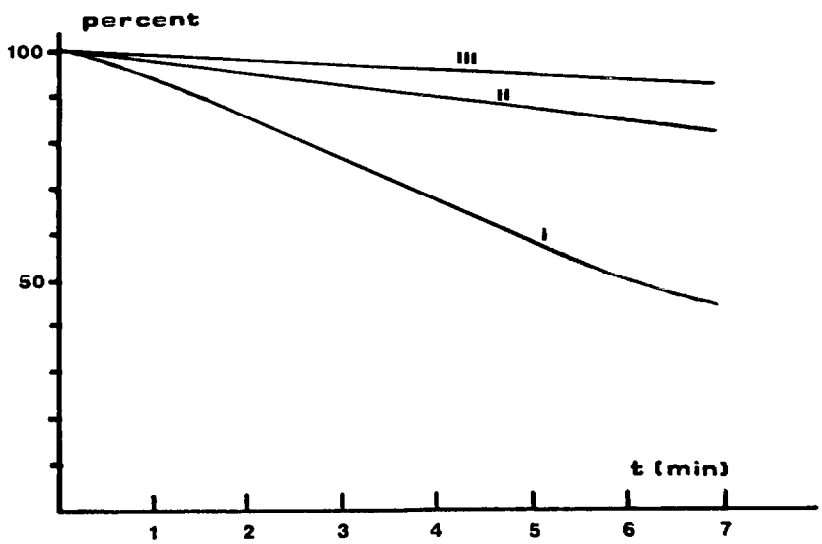

Fig. 6. Loss of fluorescence intensity for compounds I, II and III during irradiation.

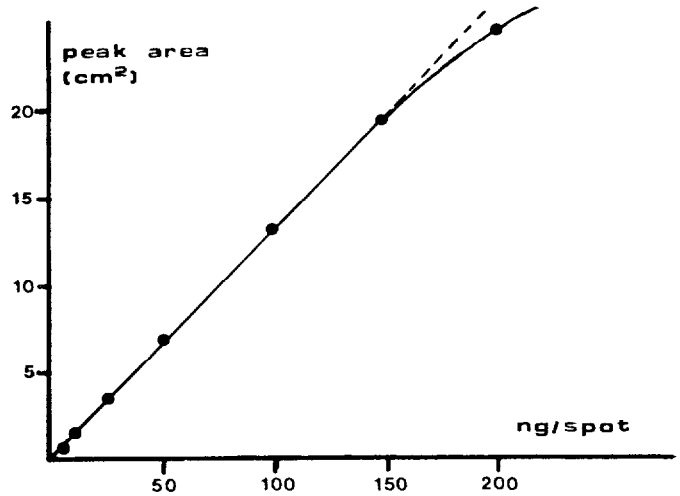

Fig. 7. Calibration curve for compound I.

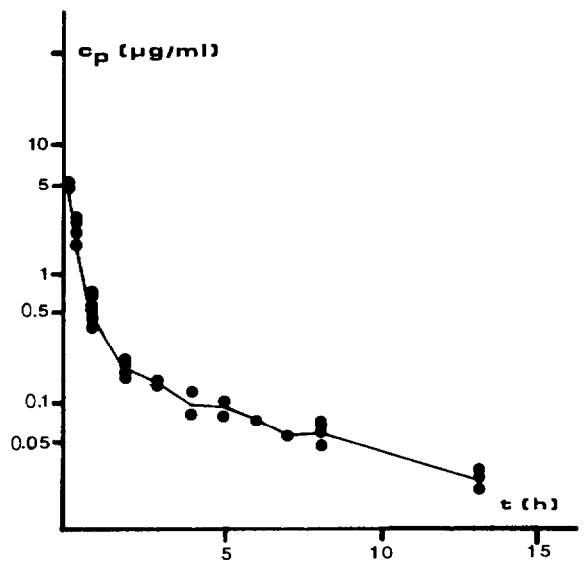

Fig. 8. Plasma levels of compound I in mice after intraperitoneal administration of 10 mg/kg.

tissues. For II, these limits were 50 ng/ml in plasma and 200 ng/ml (or ng/g) in urine, faeces and tissues. In all cases, these limits are lower than those concentrations encountered after therapeutic doses [23].

Since fluorescent compounds are unstable during irradiation, caution has been taken not to expose them to light for too long. Dependency of fluorescence intensity on the time of irradiation is shown in Fig. 6. Although appropriate stability is assured for 1 min, care was taken not to expose the spots for longer than 10 s to excitation light, in order to prevent decomposition.

The TLC method presented for the fluorescent, non-labelled diamidines I, II and III has been successfully used for the determination of pharmacokinetic

parameters in experimental animals [24]; e.g., see Fig. 8 for compound I. We therefore conclude from the data presented that the reported assay is a sensitive method for the quantitative determination of fluorescent trypanocidal diamidines after administration of therapeutic doses, which has proved its reliability and sensitivity in pharmacokinetic studies with experimental animals. Moreover, this procedure could also be useful for the determination of other fluorescent, strongly basic drugs that are sparingly soluble in non-polar solvents.

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