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High-performance liquid chromatographic assay for amiodarone N-deethylation in microsomes of rat liver

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ABSTRACT

A reversed-phase high-performance liquid chromatographic assay using ultraviolet detection is described for determining the production of the major N-dealkylated metabolite of amiodarone in rat liver microsomes. The principal advantages of this method are its simple sample preparation (protein precipitation by acetonitrile), low detection limit for N-desethylamiodarone (0.05 μmol/l) and relatively short analysis time (16 min). Its analytical applicability is demonstrated by the comparison of the kinetic parameters (maximum velocity and Michaelis–Menten constant) between Sprague-Dawley and Dark-Agouti rats.

INTRODUCTION

Amiodarone [2-butyl-3-(3,5-diiodo-4β-diethylaminoethoxybenzoyl)benzofuran] (**I**) is a potent type III anti-arrhythmic drug (Fig. 1) with anti-ischaemic properties. It is widely used in Europe and the USA for the treatment of severe ventricular and supraventricular arrhythmias, especially when they are resistant to other conventional anti-arrhythmic drugs [1]. In addition, it is generally considered as the drug of choice for the treatment of re-entrant tachycardias associated with the Wolff–Parkinson–White syndrome [2]. Pharmacokinetic data [3] indicate that amiodarone is extensively distributed (volume of distribution = 5000 l), highly tissue-bound and has a prolonged elimination half-life (28 days).

A number of high-performance liquid chromatographic (HPLC) methods for the determination of amiodarone and its major lipophilic metabolite N-desethylamiodarone (NDEA) (**II**)

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(Fig. 1) have been developed for therapeutic monitoring (serum and urine). Most of them have been realized after a preliminary liquid–liquid [4–10] or liquid–solid [11,12] extraction and concentration but as the recovery of these two products is pH-dependent [10,13], some workers have proposed a simple protein precipitation [13–15] before HPLC analysis. Assays of amiodarone in tissues have also been described with multiple extraction steps [7,11,12].

To investigate *in vitro* amiodarone N-deethylation processes which could be responsible for serious side effects [16], including life-threatening pulmonary fibrosis, hepatitis and peripheral neuropathy, a simple reversed-phase HPLC method with acetonitrile precipitation has been developed to characterize amiodarone metabolism in liver microsomal fractions. Its analytical applicability is demonstrated by kinetic studies with rat microsomes.

EXPERIMENTAL

Chemicals

Amiodarone (hydrochloride) (**I**), NDEA (hydrochloride) (**II**), bis-desethylamiodarone (hydrochloride) (**III**) and an internal standard for HPLC (**IV**) (L8040, hydrochloride), were generously provided by Labaz (Sanofi Pharma, Ambarès, France) (Fig. 1). β-Nicotinamide adenine dinucleotide phosphate (sodium salt) (NADP), D,L-isocitrate (trisodium salt), isocitrate dehydrogenase (type 4) and bovine serum albumin (fraction V) were supplied by Sigma (St. Louis, MO, USA). All other chemicals were of analytical-reagent or HPLC grade.

Animal strains

Female Sprague-Dawley (SD) (Iffa Credo, L'Arbresle, France) and female Dark-Agouti (DA) rats (CNRS, Orléans, France), weighing 200 and 150 g, respectively, were used for microsomal preparations.

Tissue samples

Rat liver microsomes were prepared according to the method described by Lake [17]. Briefly, rats were decapitated and their livers were taken, weighed and perfused with cold 0.9% NaCl. The liver samples were then homogenized three times sucessfully for 30 s in ice-cold buffer (50 m*M* Tris–0.2 *M* sucrose–1m*M* EDTA, pH 7.4) using an Ultra-Turrax homogenizer set at 5000 rpm. Post-mitochondrial supernatant was prepared by centrifugation of the homogenate at 10 000 *g* (centrifuge L8-55M with rotor 50 Ti; Beckman, Gagny, France) for 20 min. The pellets were washed with 4% (w/v) pyrophosphate buffer (pH

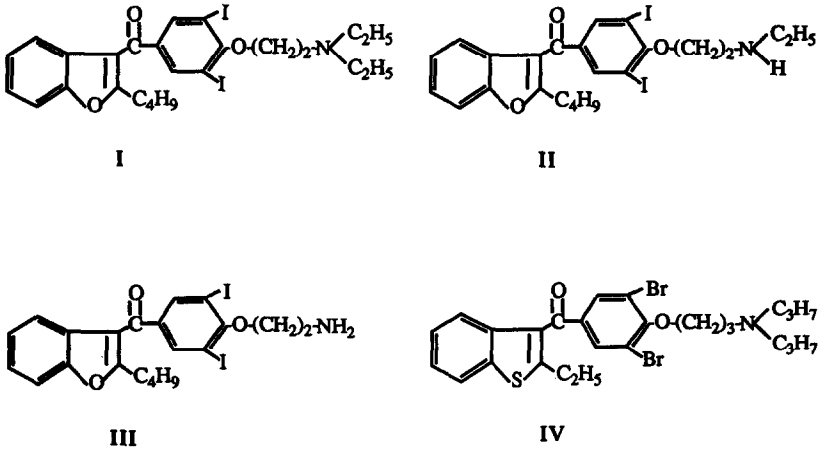

Fig. 1. Structures of amiodarone, its metabolites and the internal standard. **I** = Amiodarone, **II** = N-desethylamiodarone (NDEA), **III** = bis-desethylamiodarone and **IV** = internal standard (L8040) [2-ethyl-3-(3,5-dibromo-4γ-dipropylaminopropoxybenzoyl)-benzothiophene].

7.4) to remove non-membranous protein such as haemoglobin, and the microsomes were sedimented by ultracentrigufation twice for 60 min at 105 000 *g* with the same device. The pellets were finally resuspended in phosphate–glycerol buffer (0.1 *M* $Na_2HPO_4 \cdot 12\ H_2O$–0.01 *M* EDTA–20% glycerol, v/v; pH 7.4) and the microsomal suspensions were immediately frozen in liquid nitrogen and stored at −80°C. All manipulations were performed at 4°C.

Protein concentrations of the liver microsomes were assessed by the method of Bradford [18] on an ABA 100 analyser (Abbott, Rungis, France), with Coomassie brilliant blue in the presence of sodium dodecyl sulphate [19] using crystalline bovine serum albumin (1 mg/ml) as the standard. The assays were performed on ten-fold diluted microsomal suspensions. Total P-450 was determined by the method of Matsubara *et al.* [20] using a Uvikon 860 spectrophotometer (Kontron, St-Quentin en Yvelines, France) and according the equation of Ghersi-Egea *et al.* [21] with a molar extinction coefficient of 104 cm^{-1} mM^{-1}.

Incubation conditions and extraction procedure

For the biotransformation of amiodarone to NDEA in SD and DA rat liver microsomes, the incubation mixture consisted of microsomal proteins (0.3–2.5 mg/ml incubation) and the NADPH-generating system [22] (4 m*M* $MgCl_2$–0.7 m*M* NADP–4 m*M* isocitrate–0.7 m*M* isocitrate dehydrogenase). All reagents were dissolved in sodium phosphate buffer (0.1 *M* Na_2HPO_4, pH 7.4). After 5 min preincubation, the reaction was initiated by the addition of 50 μl of amiodarone hydrochloride in methanolic solution (0.1–6.0 g/l) to give a final range of concentrations from 3.67 to 220 μmol/l in a 2-ml total volume. In blank incubations used as controls, the NADPH-generating system, amiodarone or microsomal fractions were replaced by a corresponding volume of incubation buffer. Incubations were performed, in amber glass-capped tubes at 37°C in a shaking water-bath for 2 h.

The reaction was stopped by the addition of acetonitrile in which the HPLC internal standard (L8040) was dissolved at a concentration of 1.25 μg/ml. The contents were vigorously mixed for 30 s to allow protein precipitation. Each tube was then centrifuged at 1500 *g* for 5 min and aliquots (250 μl) of the supernatant layer were analysed directly by high-performance liquid chromatography.

High-performance liquid chromatographic analysis

NDEA produced *in vitro* by microsomal fractions was quantitated by HPLC with UV detection. The samples were analysed using a Gold System liquid chromatograph (Beckman) consisting of a solvent module (Model 126), a recorder module (Model 427) and a variable-wavelength UV detector (Model 167), connected to an NEC PC 8300 computer. Separation was performed on a C_{18} reversed-phase 5-μm ODS Hypersil column (150 × 4.6 mm I.D.) (Touzart & Matignon, Vitry-sur-Seine, France) using a mobile phase consisting of methanol–water–58% ammonium hydroxide (88:10:2, v/v/v) delivered at a flow-rate of 1.8 ml/min. The detector was set at 242 nm with a sensitivity range of 0.050 a.u.f.s. Sample volumes were injected onto the column with a manual HPLC injector (Altex 210A) fitted with a 250-μl loop. All assays were performed at room temperature.

Calibration standards were prepared before each study at an appropriate dilution to deliver between 0.46 and 1.84 μmol/l NDEA in a 2-ml final volume of the incubation buffer. These standards were incubated and precipitated under the same conditions as described for the samples. Peak-area ratios of NDEA to L8040 (internal standard) were measured and the calibration graph was obtained from linear regression analysis of the peak-area ratios *versus* concentrations. The line was then used to calculate the unknown concentrations of NDEA.

Analysis of kinetic data

The maximum velocity (V_{max}) and the Michaelis–Menten constant (K_M) values were evaluated by least-squares regression analysis using the Lineweaver–Burk plots. Values were expressed as

means ± standard deviations of four experiments.

One-way analysis of variance (ANOVA) was used for the comparison of kinetic data (V_{max} and K_M) between the two strains of rats (SD and DA).

RESULTS AND DISCUSSION

Chromatographic analysis

Fig. 2a is a typical chromatogram of a buffered standard solution containing 0.46 μmol/l NDEA. Fig. 2b is a chromatogram obtained from a precipitated sample before incubation and Fig. 2c shows the results after incubation of rat liver microsomes with amiodarone. Using the conditions described previously, the retention times were 5.1, 8.1 and 13.5 min for the metabolite NDEA (**II**), amiodarone (**I**) and the internal standard (**IV**), respectively. The analysis run takes about 16 min to complete. Compared with blank incubations used as controls, all chromatograms were free from endogenous interferences. Three additional peaks (1, 2 and 3) with retention times of 3.1, 3.4 and 3.9 min, respectively (Fig. 2c), were observed after incubation and did not interfere with the analysis. Peak 3 had the same retention time as the reference compound bis-desethylamiodarone (**III**) and could correspond to this metabolite of amiodarone. The bis-desethylamiodarone production in rat liver tissues has also been detected by Reasor *et al.* [23]. Peaks 1 and 2, absent on the chromatogram before in-

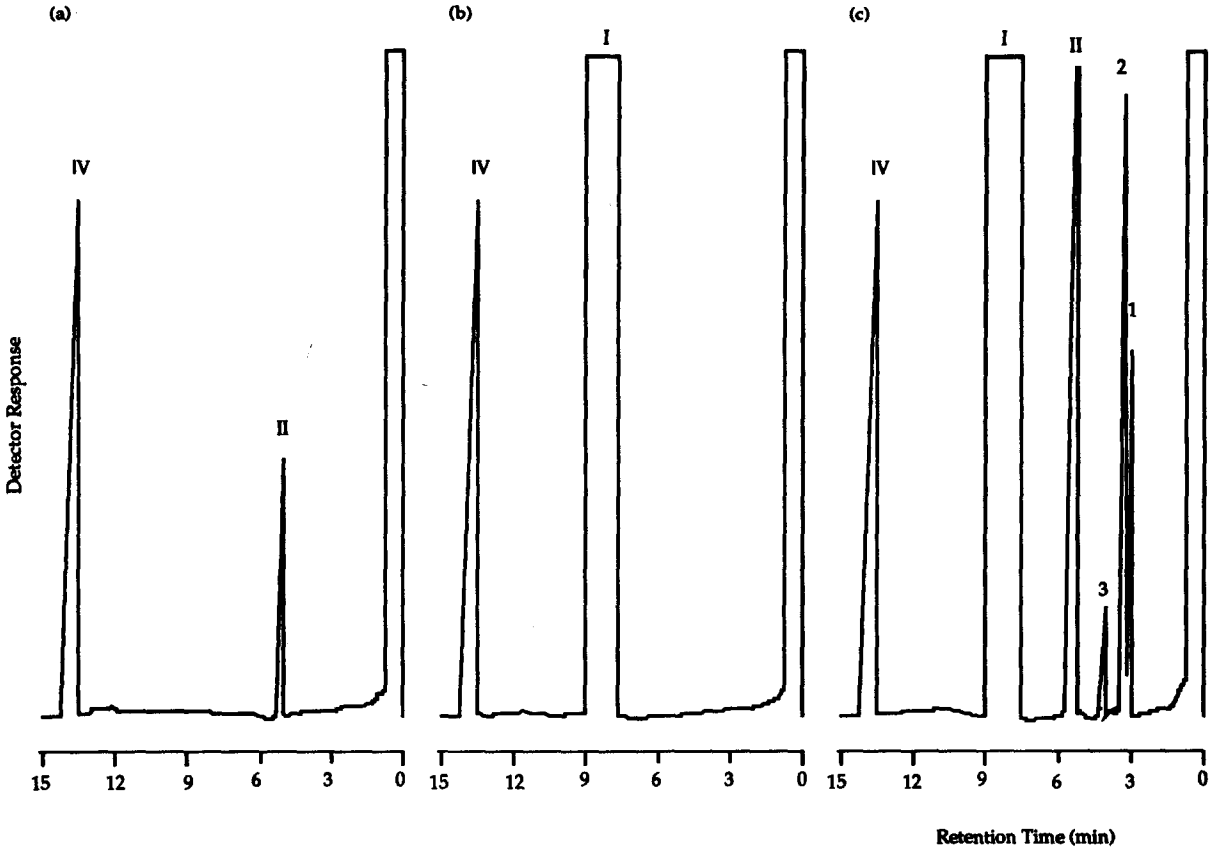

Fig. 2. Representative chromatograms of (a) a buffered standard solution containing 0.46 μmol/l NDEA (**II**), (b) a precipitated sample before incubation and (c) after incubation of SD rat liver microsomes with 73.34 μmol/l amiodarone (**I**) concentration, containing 1.25 μmol/l NDEA. **IV** = Internal standard (L8040); 3 = bis-desethylamiodarone (**III**) and 1 and 2 = unknown polar metabolites.

cubation, could correspond to other more polar metabolites.

All manipulations were carried out in amber glass-capped tubes because we had observed, in the presence of light, the formation of deiodinated compounds (monoiodoamiodarone and bis-desiodoamiodarone), the retention times of which could interfere with those of NDEA and bis-desethylamiodarone, respectively (chromatograms not shown). Moreover, the peak of monoiodoamiodarone could correspond to the endogenous substance peak described by Plomp *et al.* [13] and Ress *et al.* [9] with the acetonitrile procedure. This amiodarone photolysis in aqueous solution has previously been reported by Li and Chignell [24] and by Paillous and Verrier [25].

The calibration graph was obtained with calibration standards incubated and precipitated under the same conditions as described for the samples. Peak-area ratios of metabolite to internal standard (y) were linearly related to their concentrations (x) over the NDEA range studied (0.46–1.84 μmol/l) and up to 12 μmol/l. The linear regression equation is $y = -0.001 + 0.448x$, with the intercept not signicantly different from zero. The correlation coefficient for the regression line was 0.999.

The analytical recovery of known concentrations of NDEA (in the range 0.46–1.84 μmol/l) was determined by comparing the peak-area ratios of spiked and precipitated samples obtained without incubation with those of equivalent amounts of NDEA dissolved in acetonitrile containing the internal standard (L8040). The analytical recovery was 90 ± 5%.

For 250 μl injected onto the column, the detection limit at a signal-to-noise of 3:1 was 0.05 μmol/l for NDEA.

The intra-assay precision was established in a microsomal preparation containing the NADPH-generating system and spiked with NDEA at concentrations of 0.46 and 1.84 μmol/l. For each concentration the intra-assay precision of three consecutive runs was determined with a coefficient of variation of 9.7 and 7.0%, respectively. The inter-assay precision was determined by analysing, on three consecutive days, frozen aliquots from a similar microsomal preparation containing 1.84 μmol/l NDEA. The coefficient of variation was 3.3%.

The accuracy was defined as the overall range of mean percentage differences between three added and calculated concentrations and was 96.4 ± 6.3% for NDEA.

A simple deproteinization with acetonitrile at the pH of the incubation buffer (7.4) was chosen because the extraction techniques were time consuming with solid-phase extraction [11,12] and unclear. Indeed, many buffers such as acetate at pH 3.0 [5], aceto-acetate at pH 5.5 [6] or phosphate at pH 4.35 [10] or 7.0 [8] have been published with different extraction solvents such as diisopropylether [4,10], diethyl ether [5], hexane [6,8] or *tert.*-butyl ether [7] giving different final analytical recoveries for amiodarone, NDEA and the internal standard [9]. Moreover, if the pH of the extraction buffer with the same solvent is increased from 4.0 to 7.0, the recovery of amiodarone decreases from 103 to 81% and that of NDEA increases from 70 to 92% [10]. This observation is supported by the preliminary experiments of Flanagan *et al.* [4], indicating pH 7.4 as the optimum for the extraction of NDEA.

Under the conditions described here, this HPLC method allows the determination of NDEA produced in rat liver microsomal fractions and can be used for the simultaneous detection of other amiodarone metabolites such as bis-desethylamiodarone.

Optimum incubation conditions

The production of NDEA by DA rat liver microsomes, investigated with 36.67 μmol/l amiodarone concentration and 0.72 mg/ml of microsomal proteins, increased up to 90 min with no deviation from linearity. The equation obtained from least-squares regression analysis of the data was $y = 0.045 + 0.066x$ ($r = 0.998$, $p < 0.001$). With a 60-min incubation time and a 36.67 μmol/l substrate, the reaction was linear with protein concentrations up to 2.5 mg/ml incubation. The linear regression equation was $y = -0.226 + 6.783x$ ($r = 0.999$, $p < 0.001$). Using SD rat liver microsomes, similar results were obtained (data not shown).

The low detection limit of the HPLC analysis allows the use of (i) small microsomal protein concentrations (*i.e.* a decrease in the number of rats killed) and (ii) small amounts of amiodarone. In consequence, we have studied NDEA formation by liver microsomes with protein concentrations of 0.5 mg/ml and with an incubation time of 60 min.

Cofactor requirements

The NDEA production was evaluated in the absence of each reaction product. No metabolite peak was detected when the microsomal proteins, substrate, or NADPH were omitted from the incubation.

Reproducibility of the enzymatic reaction

Reproducibility of the enzymatic reaction was investigated four times for different microsomal fractions of the same rat livers, at 73.34 μmol/l substrate and 0.5 mg/ml of protein concentration. The coefficients of variation were 3.1% for SD and 6.3% for DA rats.

Kinetic studies

Production of NDEA by SD and DA rat hepatic microsomes, at 0.5 mg/ml of microsomal proteins, was determined with various concentrations of amiodarone from 3.67 to 220 μmol/l. Fig. 3 shows the mean results obtained for each strain. The Lineweaver–Burk plots of experimental data (Fig. 4) showed significant correlations between 1/NDEA and 1/amiodarone concentrations ($r = 0.961$, $p < 0.001$ and $r = 0.979$, $p < 0.001$ for SD and DA rats, respectively). The enzymatic activities were assumed to have monophasic Michaelis–Menten kinetics in spite of the decrease of NDEA production at 73.34 μmol/l amiodarone concentration (certainly by excess substrate inhibition).

The estimated Michaelis–Menten parameters for SD and DA rats were: $K_M = 16.45 \pm 2.91$ and 12.00 ± 0.87 μmol/l, respectively, and the corresponding $V_{max} = 3.54 \pm 0.66$ and 3.56 ± 0.30 nmol/mg protein/h, respectively.

One-way ANOVA used for the comparison of kinetic data did not show statistical differences

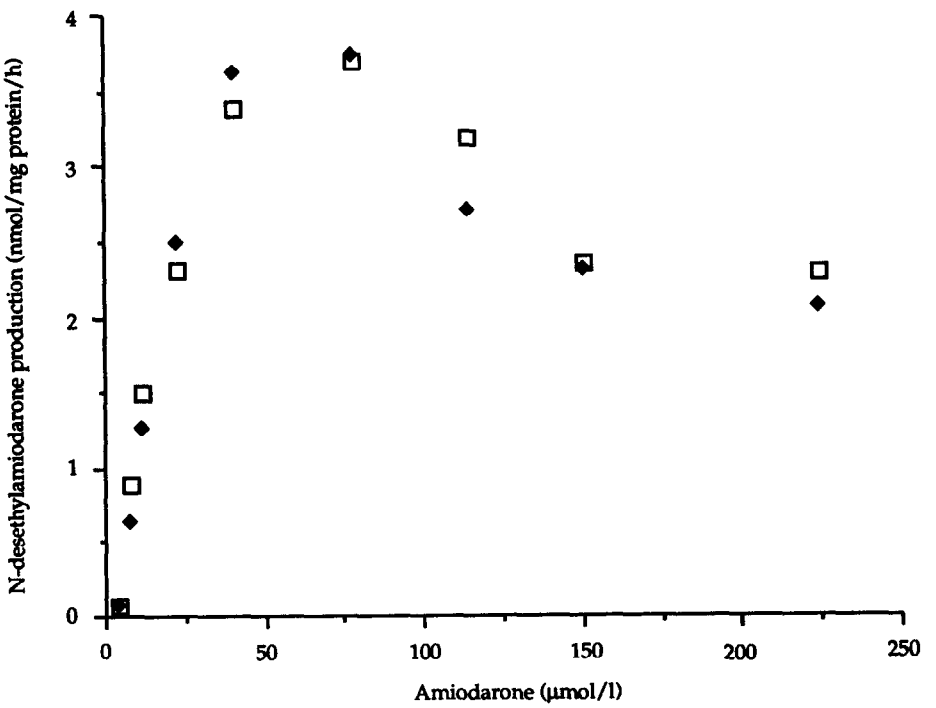

Fig. 3. Effect of various amiodarone concentrations (3.67–220 μmol/l) on the NDEA production in rat liver microsomes. Protein concentration, 0.5 mg/ml; incubation time, 60 min. □ = Dark-Agouti rats; ◆ = Sprague-Dawley rats.

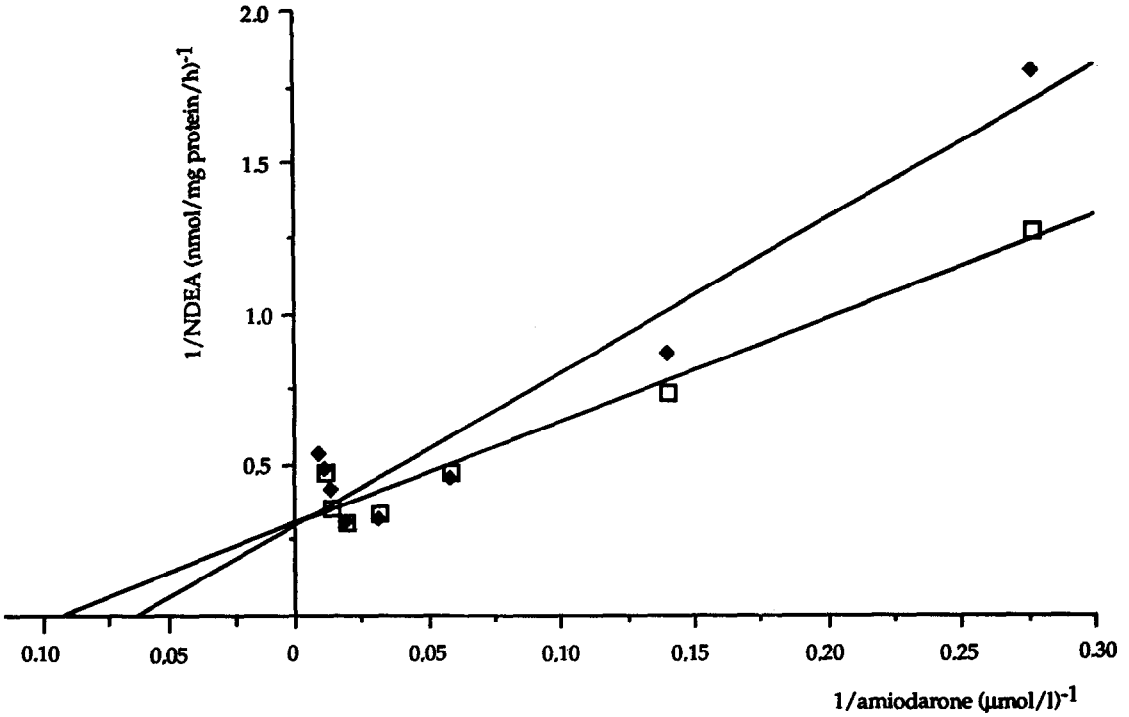

Fig. 4. Lineweaver–Burk plots of the NDEA production in microsomal fraction from rat livers. The linear regression equations are $y = 0.280 + 5.065x$ and $y = 0.278 + 3.385x$ for SD and DA rats, respectively. □ = Dark-Agouti rats; ◆ = Sprague-Dawley rats.

for the V_{max} and K_M values between the two strains.

CONCLUSIONS

The HPLC assay described here is simple and shows relatively good reproducibility, sensitivity and selectivity provided that all manipulations are performed in amber glass-capped tubes. It allows the kinetic characterization of the N-deethylation of amiodarone by rat liver microsomes and could be applied to microsomal fractions prepared from human livers to investigate the biotransformation of amiodarone.

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REFERENCES

1 J. J. Heger, E. N. Prystowsky and D. P. Zipes, *Am. Heart J.*, 106 (1983) 887.
2 S. Nattel and M. Talajic, *Drugs*, 36 (1988) 121.
3 D. W. Holt, G. T. Tucker, P. R. Jackson and G. C. A. Storey, *Am. Heart J.*, 106 (1983) 840.
4 R. J. Flanagan, G. C. A. Storey and D. W. Holt, *J. Chromatogr.*, 187 (1980) 391.
5 M. Stäubli, J. Bircher, R. L. Galeazzi, H. Remund and H. Studer, *Eur. J. Clin. Pharmacol.*, 24 (1983) 485.
6 J. M. Failler, R. Farinotti and A. Dauphin, *J. Pharm. Clin.*, 4 (1985) 415.
7 R. Kannan, S. Miller, V. Perez and B. N. Singh, *J. Chromatogr.*, 385 (1987) 225.
8 M. Lesne and P. L. Pellegrin, *J. Chromatogr.*, 415 (1987) 197.
9 K. Ress, H. M. Liebich, B. Krämer, O. Ickrath, T. Risler and L. Seipel, *J. Chromatogr.*, 417 (1987) 465.
10 Ching-Nan Ou, C. L. Rognerud, Ly T. Duong and V. L. Frawley, *Clin. Chem.*, 36 (1990) 532.
11 J. A. Menius, D. J. Schumacher, E. A. Hull-Ryde, C. Y. Leung, R. G. Cummings and J. E. Lowe, *J. Liq. Chromatogr.*, 10 (1987) 2625.
12 M. J. Moor, P. A. Wyss and M. H. Bickel, *J. Chromatogr.*, 431 (1988) 455.
13 T. A. Plomp, M. Engels, E. O. Robles de Medina and R. A. A. Maes, *J. Chromatogr.*, 273 (1983) 379.

14 S. J. Weir and C. T. Ueda, *J. Pharm. Sci.*, 74 (1985) 460.
15 C. Pham Huy, S. Costa, S. Nany, J. R. Claude and M. Hamon, *Anal. Lett.*, 24 (1991) 603.
16 J. S. Wilson and Ph. J. Podrid, *Am. Heart J.*, 121 (1991) 158.
17 B. G. Lake, in K. Snell and B. Mullock (Editors), *Biochemical Toxicology. A Practical Approach*, IRL Press, Oxford, 1987, p. 183.
18 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
19 J. M. Perini, Ch. Mizon, B. Dehon, P. Fialdes, I. Lefebvre, J. Mizon and Ph. Roussel, *Clin. Chim. Acta*, 143 (1984) 321.
20 T. Matsubara, M. Koike, Y. Tochino and K. Sugeno, *Anal. Biochem.*, 75 (1976) 596.
21 J. F. Ghersi-Egea, B. Walther, A. Minn and G. Siest, *J. Neurosci. Methods*, 20 (1987) 261.
22 F. Broly, C. Libersa and M. Lhermitte, *Drug Metab. Dispos.*, 18 (1990) 362.
23 M. J. Reasor, C. L. Ogle and S. Kacew, *Toxicol. Appl. Pharmacol.*, 97 (1989) 124.
24 A. S. W. Li and C. F. Chignell, *Photochem. Photobiol.*, 45 (1987) 191.
25 N. Paillous and M. Verrier, *Photochem. Photobiol.*, 47 (1988) 337.