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# Studies on the pharmacological properties of novel arylene bis(methylketone) compounds using solid-phase extraction and high-performance liquid chromatography

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**Abstract**

A method utilising solid-phase extraction followed by high-performance liquid chromatography has been developed to quantify novel arylene bis(methylketone) chemotherapeutics present in biological samples. The samples are extracted over cyanopropylsilane solid-phase extraction cartridges using 10 m*M* heptanesulfonate–10 m*M* tetramethylammonium chloride–4.2 m*M* $H_3PO_4$–95% $CH_3CN$ as the eluent. Analytical chromatography utilises a diisopropyl-$C_8$ reversed-phase column and a 7.5–45% $CH_3CN$ gradient in 10 m*M* heptanesulfonate–10 m*M* tetramethylammonium chloride–4.2 m*M* $H_3PO_4$–$H_2O$. Detection was by ultraviolet spectrophotometry at 300 or 240 nm. The linear response of the assay was found to extend from at least 100 μg/ml down to 97.66 ng/ml for a 100 μl injection. The assay system was utilised to determine the plasma kinetics of the compounds in mice, where all the drugs were found to display rapid absorption and elimination following intraperitoneal dosing. In vitro and in vivo studies of metabolism demonstrated that each of the compounds produced several metabolites, and that this conversion could be extensive in vivo.

*Keywords:* Arylene bis(methylketone) compounds

## 1. Introduction

The increasing, world-wide pandemic of acquired immunodeficiency syndrome (AIDS), and the increasing spread of drug-resistant *Plasmodium falciparum* malaria represent two of the world's most important causes of mortality and morbidity. It is estimated that 14 million people are infected with HIV [1], and 300 million people per year are infected with malaria [2]. Novel, effective chemotherapeutic agents are needed for use against both of these important, infectious diseases. Recently, a novel arylene bis(methylketone) compound (I, see Fig. 1) was shown to have potent activity in preventing the replication of the human immunodeficiency virus in non-replicating cells such as macrophages [3]. In addition I was found to have potent antimalarial activity against *P. falciparum* in vitro and *P. berghei* in vivo [4].

The results of these previous studies have led to an interest in studying the in vivo and in vitro pharmacological properties of both I and structural analogues. In the present study, we report the development of an accurate and sensitive method for quantifying the arylene bis(methylketone)s in biological

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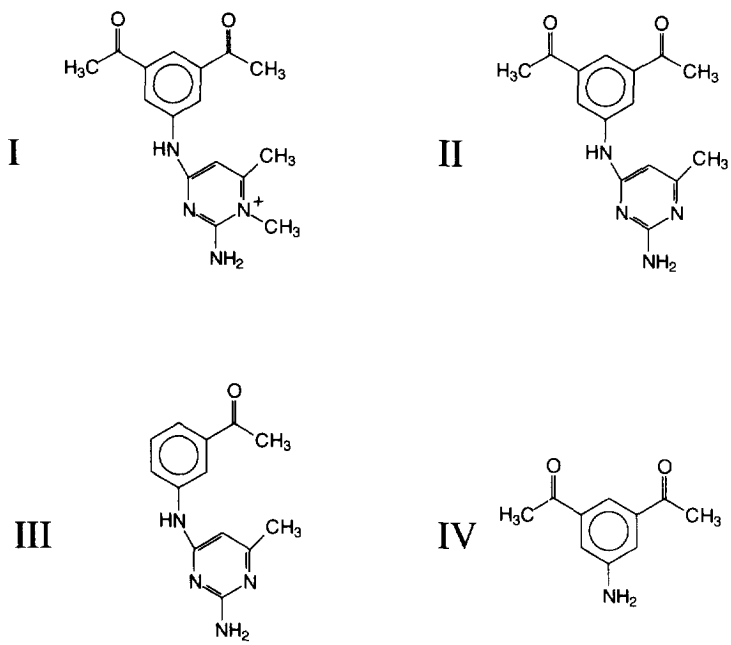


Fig. 1. Structures of the compounds used in this study. (I) 2-amino-4-(3,5-diacetylphenyl)amino-1,6-dimethylpyrimidinium chloride (CNI-H0294); (II) 2-amino-4-(3,5-diacetylphenyl)amino-6-methylpyrimidine (CNI-H1194); (III) 2-amino-4-(3-acetylphenyl)amino-6-methylpyrimidine (CNI-H1594); (IV) 3,5-diacetylaniline (CNI-H1894).

samples. In addition, this method has been used in initial investigations of the plasma pharmacokinetics and metabolism of the compounds.

## 2. Experimental

### 2.1. Chemicals

Tetramethylammonium chloride, dimethyl sulfoxide, and phosphoric acid were obtained from Aldrich (Milwaukee, WI, USA), and heptane sulfonate, NADPH, peanut oil, and heparin from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was acquired from Fisher Scientific (Fairlawn, NJ, USA), and all water was deionised through a Picopure purification system (Hydro Service and Supplies, Research Triangle Park, NC, USA). Pentamidine was obtained from May and Baker (now Rhone-Poulenc, Dagenham, UK). All of the arylene bis-(methylketone)s (see Fig. 1) were synthesized as described [4] and the purity confirmed by elemental analysis, $^1$H-NMR and melting point.

### 2.2. Sample preparation

Standard addition curves for each test compound were constructed by adding increasing amounts of drug to 1.0 ml of mouse or human $A^+$ plasma (Long Island Blood Services, Melville, NY, USA). An equal volume of 10 m*M* tetramethylammonium chloride–10 m*M* heptane sulfonate–4.2 m*M* $H_3PO_4$ (solvent A) was added to the plasma sample, which was then mixed and loaded onto a conditioned 1 g cyanopropylsilane (or octadecylsilane for IV) solid-phase extraction cartridge (Fisher Scientific). In early experiments to compare solid-phase cartridges, 100 mg diphenylsilane, cyanopropylsilane, octylsilane, and octadecylsilane cartridges were utilised (Supelco, Bellefonte, PA, USA). The cartridges were washed with 1.0 ml of water and then eluted with 1.0 ml of 10 m*M* tetramethylammonium chloride–10 m*M* heptane sulfonate–4.2 m*M* $H_3PO_4$–95%

$CH_3CN$–5% $H_2O$ (solvent B). The eluted sample was reduced to dryness in a rotary evaporator and resuspended in 1.0 ml solvent A.

### 2.3. Chromatographic conditions

100 μl of the resuspended sample was injected onto a Hewlett-Packard 1090 HPLC (Wilmington, DE, USA) equipped with a photodiode array ultraviolet–visible spectrophotometric detector, autosampler, and Chemstation operating software. The column used was a 250×4.6 mm Zorbax RX-C8 column (Mac-Mod Analyticals, Chadd's Ford, PA, USA) kept at room temperature and run at 1.5 ml/min. The mobile phase used was solvent A and 10 m*M* tetramethylammonium chloride–10 m*M* heptane sulfonate–4.2 m*M* $H_3PO_4$–75% $CH_3CN$–25% $H_2O$ (solvent C), with all runs initiated at 10% solvent C. A linear 30 min gradient to 60% solvent C was then performed, followed by a 4 min reverse gradient to initial conditions. Compounds I–III were detected by ultraviolet absorbance at 300 nm, IV at 240 nm, and pentamidine at 265 nm.

### 2.4. Pharmacokinetic studies

Female ND4 Swiss–Webster mice (21–24 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA) and randomly placed in groups of five in cages with free access to food and water. Each group of animals received 50 mg/kg of I, II, or IV, or 20 mg/kg of III in a volume of 0.5 ml. Compound I was administered intraperitoneally or by oral gavage as a solution in water or a suspension in 10% DMSO–peanut oil. The other compounds were administered intraperitoneally or by oral gavage as a solution in water titrated with sufficient 6 *M* HCl to dissolve the drug. At various time points, ranging from 5 min to 4 days, a single group of animals was killed by carbon dioxide inhalation and bled by cardiac puncture using heparin as an anticoagulant. The blood from the five mice was pooled and centrifuged at 14 000× *g* for 10 min. The volume of plasma was measured, an equal volume of solvent A added, and the mixture extracted and analysed as described above, except that the dried eluates were redissolved in 200 μl solvent A and 100 μl was injected onto the HPLC. As inspection of the blood concentration–time curves for a single i.p. injection showed a typical bi-phasic appearance, standard methods of pharmacokinetic measurement were employed [5]. For the pharmacokinetic values, $A$ and $B$ represent the zero time intercept of the distribution and elimination phases respectively, and $\alpha$ and $\beta$ the respective slopes of the phases multiplied by 2.303. The $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the calculated half-lives of the drug in each phase ($0.693/\alpha$ and $0.693/\beta$ respectively). The volume of distribution ($V_D$) was calculated as dose/$B$, and the total clearance ($Cl_{tot}$) as $\beta^* V_D$. $C_{max}$ and $t_{max}$ are the maximal plasma concentration and the time of this measurement.

### 2.5. Metabolic studies

Several female ND4 Swiss Webster mice were killed by carbon dioxide inhalation and the livers excised and rinsed with ice cold phosphate buffered saline (pH 7.4). The livers were minced, gently homogenised in 50 m*M* phosphate buffer (pH 7.4) with a Dounce homogeniser, and centrifuged at 9600×*g* for 20 min. The post-mitochondrial supernatant was kept, glycerol added to a final concentration of 20% (v/v), and frozen at −70°C in 1.0 ml aliquots until used. For each incubation, 1.0 ml of a 1.0 mg/ml drug solution was added to 3.0 ml of 50 m*M* phosphate buffer (pH 7.4), and 1.0 ml of post-mitochondrial supernatant. 500 μl of each incubate was then immediately transferred to an ice-cold tube to provide the zero-time sample, and additional 500 μl samples removed to ice-cold tubes at 8, 15, 30 and 60 min. An equal volume of solvent A was then added and the mixture extracted and analysed as described above. Control incubations were also performed where drug or post-mitochondrial supernatant was omitted. An incubation using pentamidine was performed to confirm microsomal activity [6]. Peaks in the test compound chromatograms which increased over time, and were not present in control samples lacking the enzyme preparation were treated as putative metabolites.

## 3. Results and discussion

In designing the chromatographic system for the arylene bis(methylketone)s, it was found necessary to

add both negatively and positively charged ion-pair agents (heptane sulfonate and tetramethylammonium chloride) to the buffers in order that the compounds eluted as sharp, well-resolved peaks under reversed-phase conditions. A sample chromatogram is shown in Fig. 2. The peak shape on the Zorbax RX-C8 column was superior to that obtained with a Supelcosil LC-18 column using the same buffer system (data not shown).

As the compounds eluted well under reversed-phase HPLC conditions, several reversed-phase solid-phase extraction matrices were examined. After loading the columns with 10 μg I in a total of 1.0 ml water, washing with 1.0 ml water, and eluting with 1.0 ml solvent B, 97% of the drug was recovered from cyanopropylsilane cartridges, 67% from diphenylsilane, 60% from octadecylsilane, and 37% from octylsilane (average of two separate runs for each). Of these reversed-phase matrices, the cyanopropylsilane was clearly superior for recovering I. However, when the experiment was repeated with the test compound diluted in human plasma, the recovery was only 31.0±7.1% ($n$=3) for the cyanopropylsilane cartridges. As it was likely that the drug was binding to the protein in the plasma, an equal volume of solvent A was added to the spiked plasma before extraction. Under these conditions 88.8±6.0% ($n$=4) of I was recovered from the plasma sample by extraction over cyanopropylsilane. The cyanopropylsilane extraction cartridges were also found to give excellent results for the other CNI compounds, with the exception of IV, which was best recovered by octadecylsilane cartridges (data not shown).

Having established both extraction and chromatographic systems, standard addition curves were constructed for each of the compounds using human plasma as the diluent. Starting with 1.0 ml of spiked plasma, and reconstitution of the lyophilised solid-phase eluate in 1.0 ml solvent A followed by a 100 μl HPLC injection, the limit of detection was found to be at least 97.66 ng/ml for each of the compounds. The standard addition curve was linear up to at least 100 μg/ml for each of the compounds, with the $r^2$ value for the curve exceeding 0.98 in every case. The intra-day variation for 10.0 μg/ml I was 6.8% ($n$=4), and the inter-day variation 11.3% ($n$=4) for 10.0 μg/ml and 23.8% for 195.3 ng/ml ($n$=3). In addition, the functional limit of detection could be easily increased by resuspending the lyophilised solid-phase extract in a volume of solvent A lower than that of the starting plasma sample.

This analytical system for the test compounds was

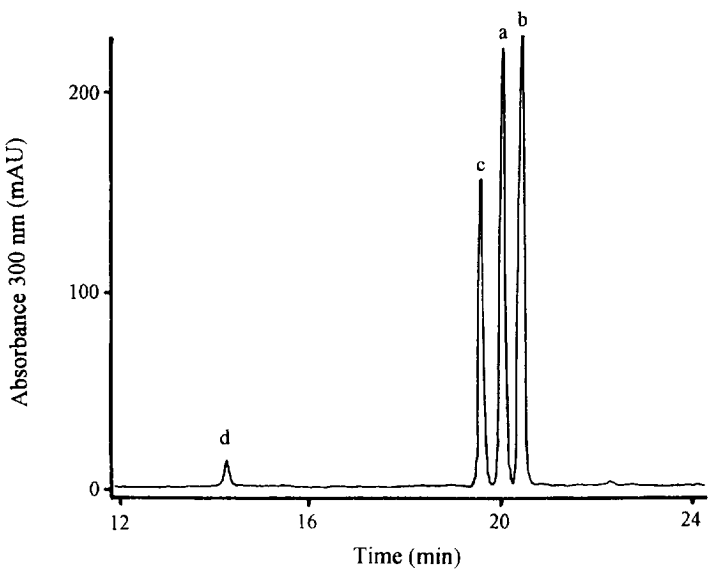


Fig. 2. Chromatographic separation of the compounds. Each compound was added to distilled water to a final concentration of 10 μg/ml and then 100 μl injected onto a Zorbax RX-C8 column using the conditions described in Section 2 and detection at 300 nm. The labels on each peak correspond to: (a) compound I; (b) II; (c) III; (d) IV. Compound IV is poorly detected at 300 nm (maximum=240 nm).

then utilised to determine the plasma concentrations following a single intraperitoneal or oral dose. As judged by the plasma concentration–time curves, each compound in the series had similar pharmacokinetic properties despite the obvious structural differences. A typical pattern is shown in Fig. 3 and the kinetic parameters are summarised in Table 1. The drugs were rapidly absorbed, with the maximal plasma concentration reached in 5–15 min, and also had a rapid distribution phase with a $t_{1/2\alpha}$ of 0.32–0.62 h. Of the analogues, I achieved the highest maximal plasma level for a single 50 mg/kg i.p. injection, with 19 μg/ml. As II had an appreciably lower maximal plasma concentration, it would appear that the presence of the methyl substituent on the heterocyclic nitrogen enhances drug absorption from the peritoneum. A comparison of II and III showed that the number of acetyl groups had little effect on drug absorption. All of the compounds, with the exception of IV, were undetectable in plasma after 24 h, and approached the limit of detection after 5–6 h. Therefore, as a general property, the CNI compounds are absorbed and eliminated rapidly.

During the analysis of the plasma samples, a number of additional HPLC peaks were detected which increased and decreased over time. Extra peaks of this nature were seen in samples from each compound in the series and examples are shown in Fig. 4. As it was possible that these peaks represented metabolites of the test compounds, the drugs were screened in a simple model of primary metabo-

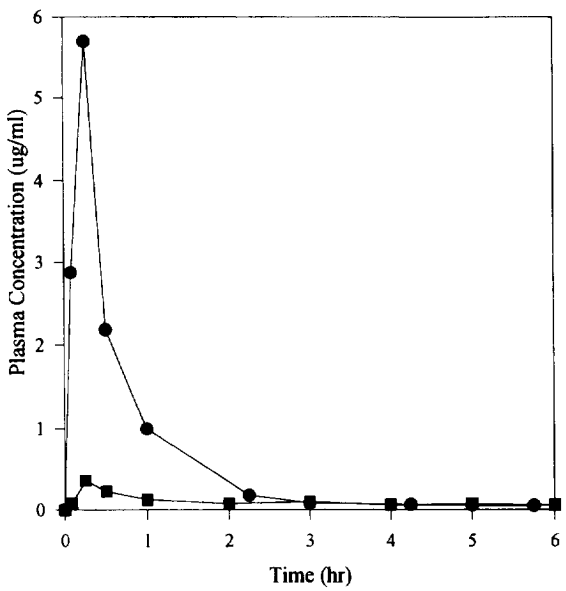


Fig. 3. Representative plasma concentrations over time in mice treated with II. Female ND4 Swiss–Webster mice were given a single 50 mg/kg injection intraperitoneally (circles) or orally (squares). At selected time points, blood was collected, extracted, and analyzed as described in Section 2. The calculated plasma concentration, in μg/ml, was then plotted against the time of sampling.

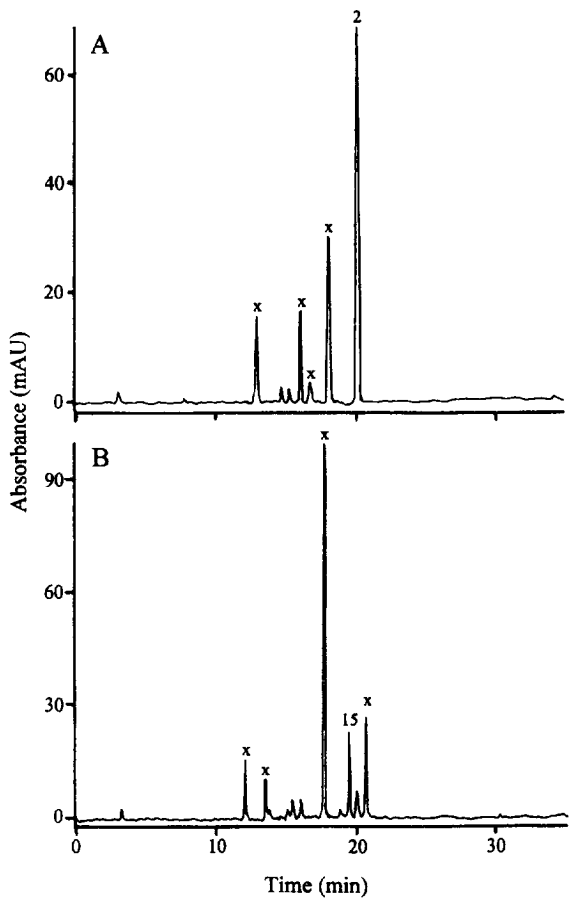


Fig. 4. Chromatograms of plasma extracts from animals treated with I or III. Female ND4 Swiss–Webster mice were given a single i.p. injection of 50 mg/kg I (A) or 20 mg/kg III (B), and blood was collected, extracted, and analyzed at various time points as described in Section 2. The chromatogram shown for I was from the 2 h time point, and that for III for the 1 h time point. The peaks labeled 2 and 15 are the parent peaks for I and III respectively. The other peaks in the chromatogram represent possible metabolites (labeled x) and endogenous plasma peaks.

Table 1
Pharmacokinetic parameters of the arylene bis(methylketone) compounds

| | I | | | II | | III | IV |
|---|---|---|---|---|---|---|---|
| Route of injection | i.p. | i.p. | Oral | i.p. | Oral | i.p. | i.p. |
| Dose (mg/kg) | 50 | 50 | 50 | 50 | 50 | 20 | 50 |
| Vehicle | DP[a] | W[a] | DP | W | W | W | W |
| AUC (μg*h/ml) | 9.2 | 8.8 | 0.6 | 3.9 | 0.6 | 0.8 | 20 |
| $C_{max}$ (μg/ml) | 19 | 19 | 0.4 | 5.7 | 0.4 | 1.9 | 13 |
| $t_{max}$ (min) | 5 | 5 | 60 | 15 | 15 | 15 | 5 |
| $\alpha$ ($h^{-1}$) | 1.1 | 1.7 | – | 1.8 | – | 2.1 | 1.2 |
| $\beta$ ($h^{-1}$) | 0.2 | 0.2 | – | 0.2 | – | 0.04 | 0.03 |
| $A$ (μg/ml) | 14 | 16 | – | 5.2 | – | 1.1 | 15 |
| $B$ (μg/ml) | 0.1 | 0.1 | – | 0.1 | – | 0.01 | 0.2 |
| $t_{1/2\alpha}$ (h) | 0.6 | 0.4 | – | 0.4 | – | 0.3 | 0.6 |
| $t_{1/2\beta}$ (h) | 4.6 | 3.7 | – | 3.7 | – | 17 | 23 |
| $V_D$ (l) | 14 | 20 | – | 5.2 | – | 40 | 6.6 |
| $Cl_{tot}$ (ml/min) | 35 | 63 | – | 17 | – | 26 | 3.3 |
| Bioavailability | – | – | 0.06 | – | 0.15 | – | – |

[a] DP=DMSO/peanut oil; W=water.

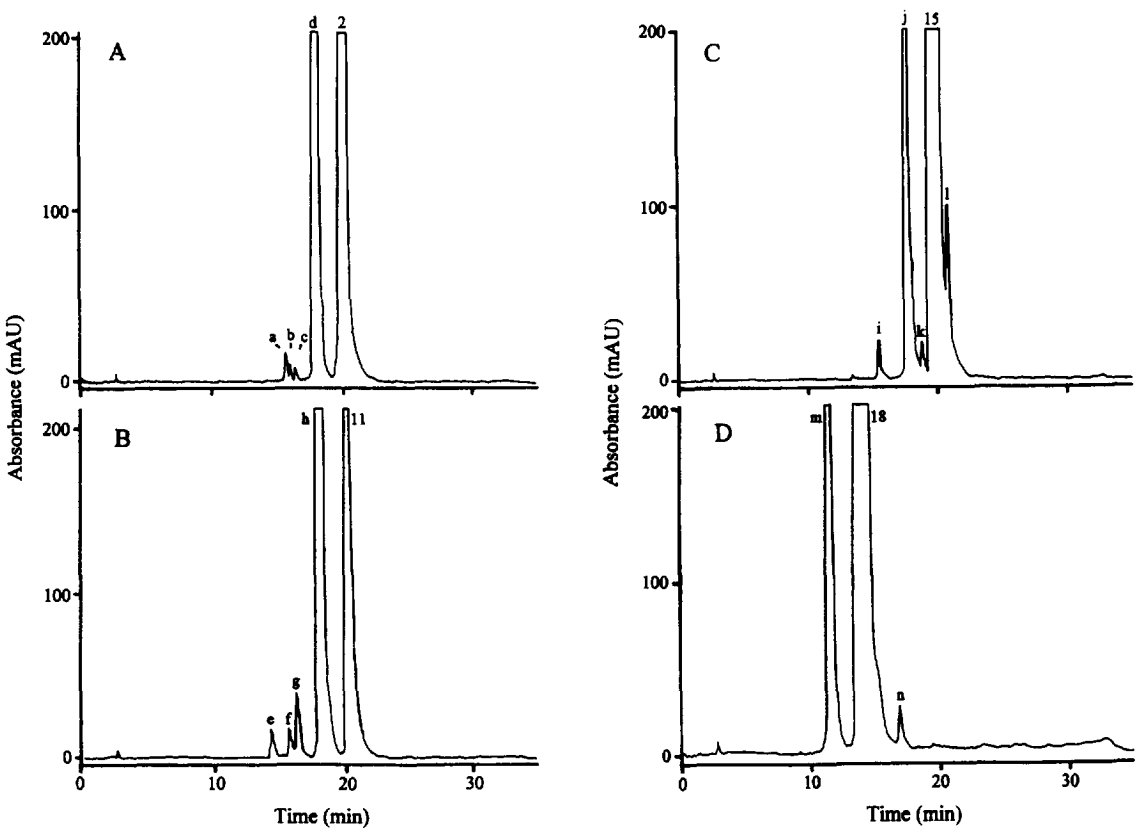


Fig. 5. In vitro metabolism of the test compounds. The drugs were incubated with mouse liver post-mitochondrial supernatants and NADPH for various lengths of time, and extracted and analyzed as described in Section 2. The chromatograms shown are from the 60 min time point for (A) I, (B) II, (C) III, and (D) IV. The peaks labeled 2, 11, 15, 18 refer to the parent compound peaks, and those labeled a–n to putative metabolite peaks that increased over time and were not present in control incubations. All off-scale peaks were single peaks, and the scale was chosen to allow presentation of trace metabolite peaks.

lism. Using post-mitochondrial supernatants of homogenised mouse livers as a source of enzyme, the drugs were incubated in the presence of NADPH. Pentamidine was utilised as a positive control, and the seven expected, primary metabolites were detectable [6], confirming the activity of the enzyme preparation (data not shown). Extraction and HPLC analysis of incubates containing the test compounds showed the presence of numerous putative metabolite peaks that were not present in negative controls (Fig. 5). Incubation of I, II, or III produced one major and three minor metabolites, and IV produced one major and one minor metabolite. The major metabolite was found to elute 0.9–1.2 min closer to the solvent front for I, II, and III, suggesting that the same position was being altered in each of these compounds. Mass spectrometric identification of the metabolites is hampered by the presence of the non-volatile ion-pair agents present in the HPLC buffers, and we are currently working on methods to circumvent this problem.

The metabolic conversion in vitro was considerable, with 43% of I, 65% of II, 12% of III and 17% of IV altered during the course of a 60 min incubation (as judged by peak area). These results indicated that appreciable metabolism could occur in vivo.

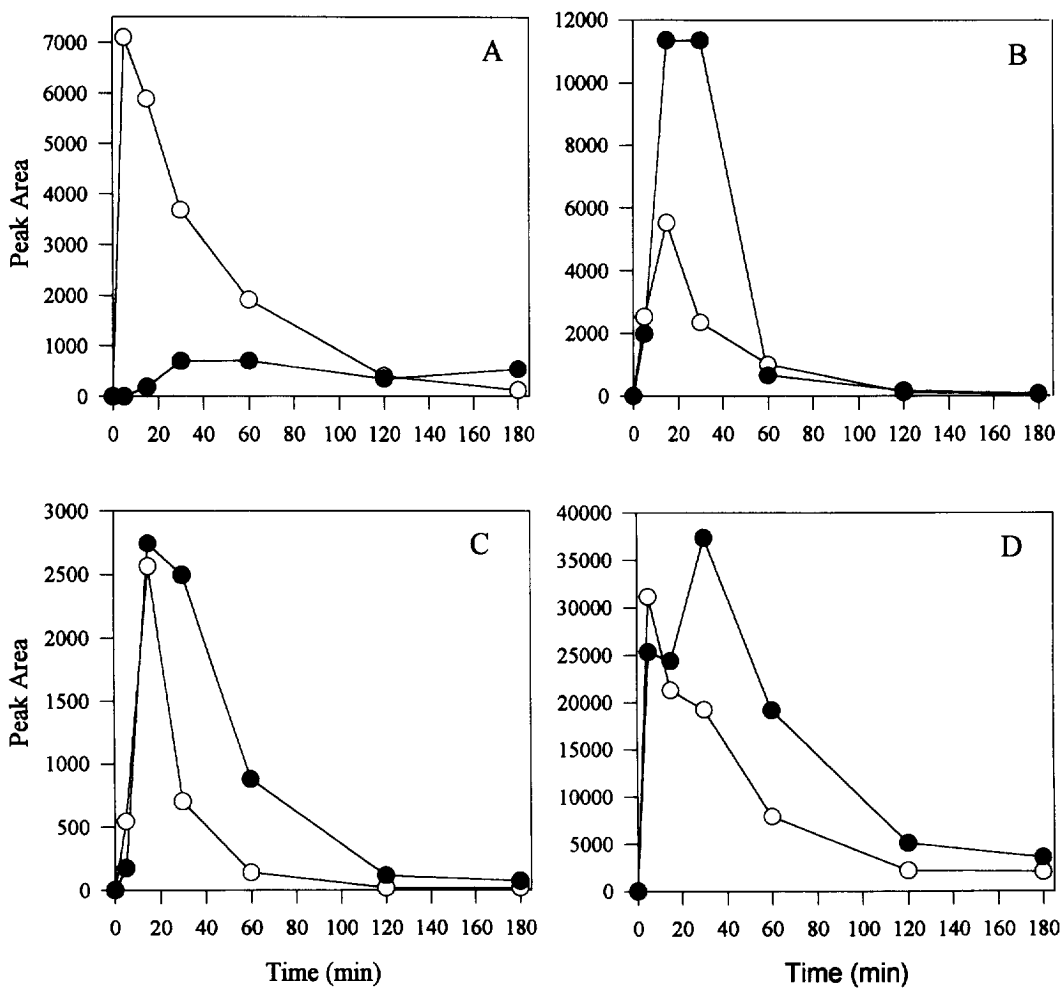


Fig. 6. In vivo metabolism of the compounds. Female ND4 Swiss–Webster mice received a single intraperitoneal dose of (A) 50 mg/kg I, (B) 50 mg/kg II, (C) 20 mg/kg III, or (D) 50 mg/kg IV. At the indicated time points, blood samples were collected, extracted, and analyzed as described in Section 2. In each case, the peak area for the parent compound (open circles) and the combined peak area for all the putative metabolites (closed circles) are plotted against time after dosing.

Re-examination of the plasma samples confirmed that several of the unknown plasma peaks seen in Fig. 4 corresponded to the putative metabolites in Fig. 5. However, the metabolic model system did not produce all of the unknown peaks seen in the plasma samples. In particular, a plasma peak eluting at 11–14 min was seen with all the compounds in vivo, but not seen at all in the in vitro test system or in plasma from untreated animals. With the exception of I, there appeared to be a large amount of metabolic conversion in vivo (Fig. 6), and the metabolites were found to also have very rapid plasma kinetics.

Of the compounds studied to date, I possesses the best characteristics, with the highest anti-HIV and anti-malarial activities [3,4], and very low toxicity [4]. The present results indicate that the compound is very rapidly absorbed and eliminated, but has fairly low bioavailability. The solid-phase extraction and HPLC method outlined here has been found to be sensitive and accurate, and has found utility in monitoring drug purity during synthesis, stability in solution, pharmacokinetics and metabolism.

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