Journal of Chromatography, 430 (1988) 429–437
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4261

Note

High-performance liquid chromatographic assay for the benzotriazine di-N-oxide (SR 4233) and its reduced metabolites in biological materials

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(First received January 11th, 1988; revised manuscript received April 18th, 1988)

It is widely believed that the presence of radioresistant hypoxic cells in human solid tumours may limit their curability by radiotherapy [1]. Consequently it has been proposed that the combination of radiotherapy with drugs which are preferentially cytotoxic to hypoxic cells may improve local tumour control [2].

Two important classes of compounds are currently known to show enhanced toxicity under hypoxic conditions, namely the nitroimidazole hypoxic cell sensitizers and the quinone bioreductive alkylating agents such as mitomycin C [3]. The cytotoxicity of these agents is thought to involve their reductive metabolism to highly reactive species, a hypoxia-facilitated process which can be catalysed by a variety of reductases [4, 5]. These drugs are 5–80 times more toxic under hypoxic conditions (see ref. 6). More recently a new class of hypoxic cell cytotoxins, the benzotriazine N-oxides, has been discovered. The lead compound, 3-amino-1,2,4-benzotriazine-1,4-dioxide (I, Fig. 1) is up to 200 times more toxic towards hypoxic compared to aerobic mammalian cells in vitro [6]. Previous studies have implicated reductive metabolism in the mode of action of I though the precise enzymology of this reaction is unclear [6, 7].

In order to study the enzymology, pharmacokinetics and metabolism of this drug we have developed two sensitive high-performance liquid chromatographic (HPLC) assays for I and its reduction products II and III (Fig. 1) in biological materials. One method employs an N-oxide as an internal standard and is suitable for use in anaerobic microsomal metabolism experiments in vitro, while the other uses a nitroimidazole as the internal standard and is suitable for pharmacokinetic and metabolism experiments in vivo. The relative merits of both assays are discussed.

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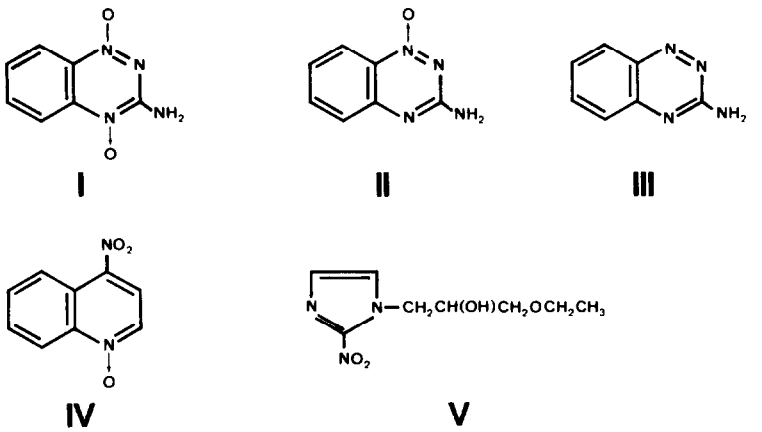

Fig. 1. Structures of (I) 3-amino-1,2,4-benzotriazine-1,4-dioxide, (II) 3-amino-1,2,4-benzotriazine-1-oxide, (III) 3-amino-1,2,4-benzotriazine, (IV) 4-nitroquinoline N-oxide and (V) 1-(2-nitroimidazoyl-1-yl)-3-ethoxypropan-2-ol.

EXPERIMENTAL

Materials

3-Amino-1,2,4-benzotriazine-1,4-dioxide (I; SR 4233) was obtained from Dr. T.A. Strike of the U.S. National Cancer Institute (Bethesda, MD, U.S.A.), 3-amino-1,2,4-benzotriazine-1-oxide (II; SR 4317) from Dr. W.W. Lee of Stanford Research International (Menlo Park, CA, U.S.A.) and 3-amino-1,2,4-benzotriazine (III; SR 4330) from Dr. P. Wardman of the CRC Gray Laboratory (Northwood, U.K.). The 2-nitroimidazole internal standard 1-(2-nitroimidazoyl-1-yl)-3-ethoxypropan-2-ol (IV; Ro 07-0913) was supplied by Dr. C.S. Smithen of Roche Products (Welwyn Garden City, U.K.) and the N-oxide internal standard 4-nitroquinoline N-oxide was purchased from Sigma (Poole, U.K.). Structures are shown in Fig. 1. Methanol (HPLC grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Water was distilled once and then deionised using a four-cartridge Milli-Q water purification system (Millipore, Molsheim, France). Mobile phases were passed through 0.45-μm Millipore filters and thoroughly degassed under vacuum before use.

Spectrophotometry

Samples of I, II and III in methanol (5 $\mu g\ ml^{-1}$) were scanned from 350 to 200 nm using a Model 25 spectrophotometer connected to a Series B controller–recorder (Beckman Instruments, Irvine, CA, U.S.A.). Quartz semi-microcuvettes with a 1 cm light path length were employed.

Drug administration and animals

Adult inbred C3H/He mice of both sexes were obtained from OLAC (Bicester, U.K.) and our own breeding colony. Mice were allowed laboratory chow and water at libitum and were used at 25–30 g body mass. RIF-1 tumours were grown intramuscularly in the hind leg as previously described [8].

Compound I was dissolved in dimethylsulphoxide (DMSO) and then diluted 1:6 with phosphate-buffered saline (PBS, pH 7.4) before intraperitoneal (i.p.) injection in a volume of 0.01 ml g^{-1} body mass at a dose of 0.2 mmol kg^{-1} (35 μg g^{-1}). Animals were sacrificed 30 min after i.p. injection. Whole blood was removed under diethyl ether anaesthesia by cardiac puncture into heparinised syringes and centrifuged at 3000 *g* for 15 min at 4 °C to obtain plasma. Tissues were rapidly removed and immediately snap-frozen on solid carbon dioxide. Urinary recovery experiments were carried out with four mice contained in a Urimax metabolism cage, and urine was collected for 8 h on solid carbon dioxide. Samples were handled at 4 °C and stored at −20 °C for up to three days before analysis.

Anaerobic metabolism

Mouse liver microsomes were prepared and stored using standard techniques [9]. Incubations were carried out under nitrogen using specially adapted 25-ml conical flasks as previously described [10]. Each incubation contained 0.3 mg protein, 0.9 m*M* NADPH and NADH and 100 m*M* sodium phosphate buffer (pH 7.4) in a final volume of 3 ml. Aliquots (100 μl) of this reaction mixture were removed at appropriate times through air-tight septa for HPLC analysis.

Sample preparation

The following procedure was found to be suitable for samples of urine, heparinised blood plasma and aliquots of anaerobic incubation mixtures. Each sample (100 μl) was treated with 2 vol. of methanol containing the N-oxide internal standard (IV; 4-nitroquinoline N-oxide at 25 mg l^{-1}) and mixed thoroughly. After centrifugation at 3000 *g* for 15 min at −15 °C the supernatant was removed and an aliquot (5 μl) injected into the HPLC system for analysis. This extraction technique was also found to be suitable for tissue homogenates (33%, w/v) except that the extracting methanol contained the 2-nitroimidazole V at 60 mg l^{-1} in place of 4-nitroquinoline N-oxide.

Standards were spiked with known concentrations of I, II and III and processed identically alongside test samples.

Chromatography

Chromatography was carried out using equipment and radial compression columns supplied by Waters Assoc. (Milford, MA, U.S.A.). The equipment comprised two Model 6000 A pumps, a Model M 45 pump, a Model 730 data module, a Model 720 system controller and a Model 710A WISP automated sample injector. Separations were carried out using a μBondapak phenyl Rad-Pak column (10 cm × 8 mm, 10-μm beads) under compression from a Z-module and protected by a Resolve cyanopropyl (CN) Guard-Pak precolumn. The mobile phase consisted of 33% methanol in water and was delivered isocratically at a flow-rate of 2.5 ml min^{-1}. Absorbance of the column effluent was monitored using a Model 440 fixed-wavelength detector set at 254 nm and coupled to the Model 730 data module chart recorder (chart speed 5 mm min^{-1}). Absorbance ratios were obtained using a Waters Model 490 programmable multiwavelength detector operating at 265, 254 and 240 nm and coupled to two Servoscribe chart recorders (three channels

used; chart speed 5 mm min^{-1}). Chromatography was carried out at room temperature. Quantitation was by peak-height ratio with reference to standards spiked into appropriate blank sample material.

RESULTS

Fig. 2 shows that I exhibits absorbance maxima at 265 and 210–220 nm. By comparison, for II and III absorbance increases steadily from around 300 nm to a peak at 210–220 nm with no discrete maxima between 230 and 350 nm. Consequently, routine chromatographic monitoring was carried out at an intermediate wavelength of 254 nm, at which all three compounds showed appreciable absorbance.

Chromatography was originally attempted on μBondapak octadecylsilane (C_{18}), Resolve cyanonitrile (CN) and octylsilane (C_8) columns (all 10 cm×8 mm; 10-μm bead size) but resulted in peak tailing, poor resolution of the parent compound I from the solvent front and/or of the two metabolites II and III from each other. By contrast, good separations and peak shapes were obtained with μBondapak phenyl columns.

It was noticed that certain supplies of I contained small amounts of material which co-eluted with the single N-oxide metabolite II (4–9%). Chromatographic purification of I and reinjection into the HPLC system showed no additional peaks, confirming that the extra material (II) was a contaminant and not a breakdown product. In view of this and for quality control purposes, chromatographic peaks were characterised in terms of their absorbance ratios at selected wavelengths and compared to the values obtained with authentic material. Table I summarises these absorbance ratios (265/254 and 240/254 nm) for authentic samples of I, II and III as well as for in vivo plasma and tumour samples. The

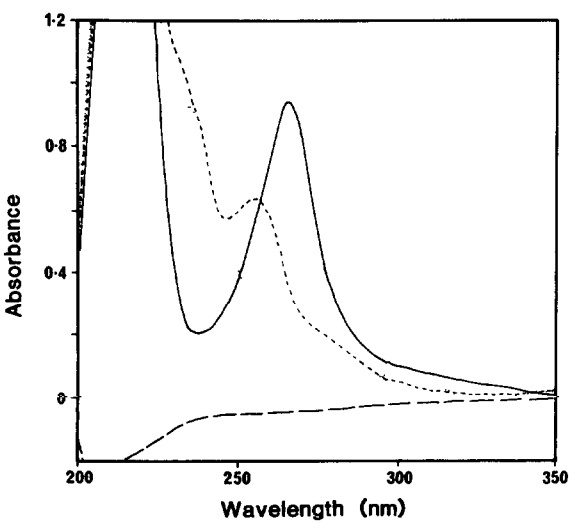

Fig. 2. UV absorbance spectra for methanol blank (– –), I (—), II (– – –) and III (•••). All compounds were dissolved in methanol (5 μg ml^{-1}) and read against a methanol reference.

TABLE I

ABSORBANCE RATIOS OBTAINED FROM CHROMATOGRAMS OF VARIOUS SAMPLES CONTAINING I, II, AND III

Ratio R_1 = 265/254 nm and ratio R_2 = 240/254 nm. Values were derived from peak heights at each specific wavelength. Results are mean ± SD for four determinations. BLD = Below limit of detection.

Sample	Compound I		Compound II		Compound III	
	Ratio R_1	Ratio R_2	Ratio R_1	Ratio R_2	Ratio R_1	Ratio R_2
Authentic material*	2.025 ±0.044	0.283 ±0.013	0.689 ±0.030	1.048 ±0.022	0.292 ±0.044	1.710 ±0.037
Plasma**	1.947 ±0.057	0.274 ±0.015	0.705 ±0.023	0.998 ±0.122	BLD	1.817 ±0.156
Tumour homogenate***	1.852 ±0.189	0.265 ±0.038	0.657 ±0.047	1.070 ±0.036	0.358 ±0.039	1.717 ±0.232

*Authentic I (batch B), II and III in methanol.
**A sample of C3H/He mouse plasma taken 30 min after 0.2 mmol kg^{-1} I i.p.
***A homogenate of a RIF-1 mouse tumour (33%, w/v) taken 30 min after 0.2 mmol kg^{-1} I i.p. (see also Fig. 5).

ratios were clearly different and characteristic for the three compounds. Values obtained for the peaks in the chromatograms of in vivo samples were in close agreement with those of the authentic material, and similar results were obtained in other chromatographic systems. In addition the nature of the minor contaminant in the I supplies was unambiguously shown to be II. Correction was made for this where appropriate.

Fig. 3a shows a chromatogram of a methanol extract of an aliquot of an anaerobic incubation mixture taken 15 min after the addition of drug vehicle (50 μl DMSO) and containing internal standard 4-nitroquinoline N-oxide (peak IV). Fig. 3b shows a chromatogram of a similar incubation extract which had been spiked with I, II and III and also containing internal standard. The chromatogram in Fig. 3c is of an aliquot from a typical anaerobic incubation mixture 15 min after the addition of I (2.0 m*M*). As well as the internal standard there is a large peak corresponding to the parent compound I and another representing the single N-oxide metabolite II; there is, however, no evidence of the benzotriazine metabolite III. Retention times were 3.4, 5.8, 7 and 12 min for I, II, III and the N-oxide internal standard (IV), respectively, and typical values for the peak-height equivalents to a theoretical plate were 0.35, 0.046, 0.063 and 0.053, respectively.

Fig. 4 shows chromatograms of pooled urine from four mice collected for 8 h after drug vehicle (a) or I (b) administration, respectively. In Fig. 4c there is a clear peak corresponding to I and two minor peaks corresponding to II and III.

The control chromatogram for the anaerobic incubation mixture (Fig. 3a) contains no interfering peaks, and similar results were obtained for plasma. Blank urine (Fig. 4a) contains a small peak which co-elutes with the minor metabolite III.

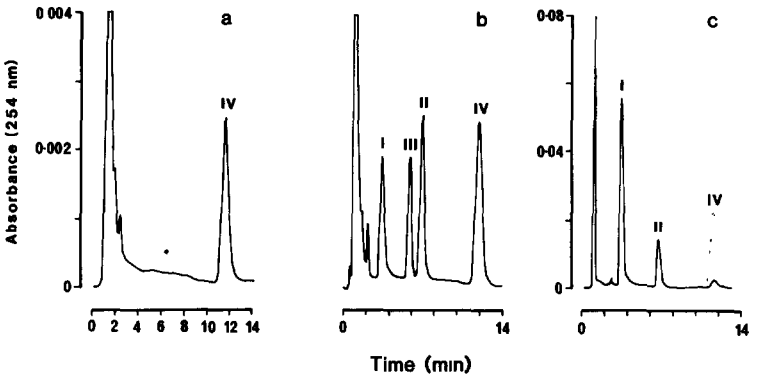

Fig. 3. Chromatograms of methanolic extracts of aliquots from anaerobic, mouse liver microsomal incubation mixtures. (a) Sample taken 15 min after the addition of drug vehicle (50 μl DMSO), showing the internal standard 4-nitroquinoline N-oxide (IV; 25 mg l^{-1} methanol). (b) A similar sample to (a) but spiked with I (9.1 μg ml^{-1}), II (10.9 μg ml^{-1}) and III (10 μg ml^{-1}) and containing the N-oxide internal standard. (c) A sample taken 15 min after the addition of 2.0 m*M* I and containing I (384 μg ml^{-1}), II (81.5 μg ml^{-1} total concentration of which 61% is metabolite) and the N-oxide internal standard, also shown detected at ten times higher sensitivity (- - -). Chromatographic conditions: column, μBondapak phenyl Rad-Pak fitted with a Resolve cyanonitrile (CN) Guard-Pak guard column; mobile phase, 33% methanol in water; flow-rate, 2.5 ml min^{-1}; column pressure, 88 bar; temperature, ambient; detection, absorbance at 254 nm; sample injection volume, 5 μl; chart speed, 5 mm min^{-1}.

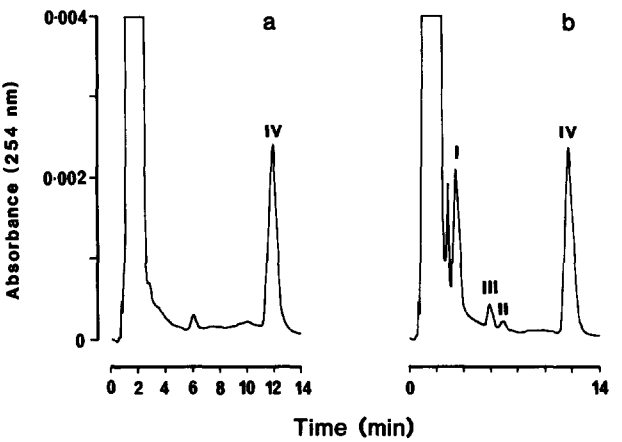

Fig. 4. Chromatograms of methanolic extracts of pooled male C3H mouse urine collected from four male mice injected i.p. 8 h previously with: (a) drug vehicle (DMSO–PBS, 1:6) and containing the N-oxide internal standard IV and a minor interfering peak; (b) 0.2 mmol kg^{-1} I and containing I (8.78 μg ml^{-1} of urine), II (0.49 μg ml^{-1} of urine) and III (0.52 μg ml^{-1} of urine) as well as the N-oxide internal standard. Chromatographic conditions as in Fig. 3.

Using this method it can be seen that all four compounds are readily resolved from each other. The efficiency of recovery from plasma and incubation mixtures was >95%. Plots of peak-height ratio (analyte/internal standard) were linear over the ranges studied (0.2–10 000 μg ml^{-1} for I, 0.2–1000 μg ml^{-1} for II and

0.2–500 $\mu g\ ml^{-1}$ for III) and had zero intercepts. The same-day coefficient of variation for six replicate analyses (10 $\mu g\ ml^{-1}$ in plasma) were 9.5, 3.7 and 2.4% for I, II and III, respectively. Allowing a signal-to-noise ratio of 2 the lower limit of detection was 0.3–0.4 $\mu g\ ml^{-1}$ for an injection volume of 5 μl. This represents an on-column injection of 0.5–0.7 ng.

Concentrations of I and its two reduced metabolites were stable in methanol extracts from all the biological materials for at least 12 h at room temperature. The internal standard 4-nitroquinoline N-oxide (IV) was also stable in methanol extracts of plasma, urine and aliquots of incubation mixtures, but was rapidly degraded in similar extracts from liver, tumour and brain tissue homogenates (33%, w/v). Consequently for in vivo studies of drug pharmacokinetics the 2-nitroimidazole V was included as an internal standard in the deproteinising methanol (60 $mg\ l^{-1}$). Tissue sample preparation and analysis was as described above and methanolic drug extracts were shown to be stable for at least 12 h at room temperature.

Fig. 5 shows chromatograms of a methanol extract of homogenate from a RIF-1 tumour which was removed from a C3H mouse 30 min after i.p. administration of either drug vehicle (a) or 0.2 mmol kg^{-1} I i.p. (b), respectively. The blank chromatogram shows the internal standard V with a retention time of 4.8 min but contains no interfering peaks. Fig. 5b contains peaks corresponding to I, II and III as well as the internal standard, all clearly resolved from each other. The overall performance of this assay was similar to the previous method employing 4-nitroquinoline N-oxide as the internal standard except that drug extraction was $\geqslant 90\%$. Calibration curves were linear up to a concentration of 500 $\mu g\ ml^{-1}$ for all three compounds. This was limited by interference in internal standard peak shape by I at higher concentrations.

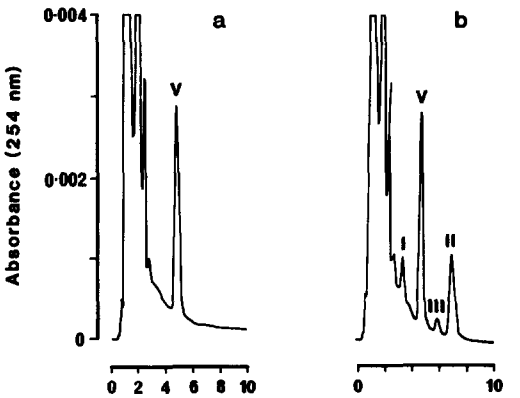

Fig. 5. Chromatograms of RIF-1 leg tumour homogenates (33%, w/v) taken from a female C3H mouse 30 min after i.p. administration of: (a) drug vehicle (DMSO–PBS, 1:6) and containing the 2-nitroimidazole internal standard V (60 $mg\ l^{-1}$ of methanol); (b) 0.2 mmol kg^{-1} I and containing I (2.04 $\mu g\ ml^{-1}$ of homogenate, 6.13 $\mu g\ g^{-1}$ of tumour), II (5.37 $\mu g\ ml^{-1}$ of homogenate, 16.1 $\mu g\ g^{-1}$ of tumour) and III (2.34 $\mu g\ ml^{-1}$ of homogenate, 7.01 $\mu g\ g^{-1}$ of tumour) and the nitroimidazole internal standard. Chromatographic conditions as in Fig. 3.

DISCUSSION

The HPLC techniques described here are both capable of quantitating I and its two reduced metabolites (II and III) at pharmacologically active concentrations in several different biological materials [6]. Excellent resolution and all round performance was achieved using a phenyl column. Although two other HPLC methods using octadecylsilane (C_{18}) columns have been reported, no details were given regarding the chromatograms obtained or the assay characteristics [6, 7]. In our laboratory C_{18} columns were found to give inferior peak shape and inadequate resolution of the parent compound benzotriazine di-N-oxide from the solvent front for use with biological specimens.

The application of absorbance ratios for quality control confirmed the identity of the peaks as I, II and III in a mouse plasma and tumour sample, as well as the nature of the minor contaminant (II) in certain batches of the parent drug (I).

4-Nitroquinoline N-oxide (IV) was chosen as an internal standard because it is an N-oxide of similar structure to I (see Fig. 1). In addition the calibration curve for this method is linear up to very high concentrations of I (56 m*M*) such as might be employed in enzyme kinetic studies in vitro. This internal standard was suitable for urinary recovery, plasma and in vitro microsomal enzyme experiments, but was unstable in methanolic tissue extracts, possibly as a result of non-enzymatic reduction as occurs with certain other N-oxides [11]. An alternative internal standard for use with tissue homogenate samples was the 2-nitroimidazole (V). While this compound is less similar in structure to the benzotriazine N-oxides (see Fig. 1), it has the advantage of being stable in extracts of all the biological materials studied and also results in a shorter overall run time for the analysis. Because of the factors discussed above we recommend the use of IV (N-oxide) for in vitro microsomal metabolism experiments and V (2-nitroimidazole) for pharmacokinetic studies in vivo.

Chromatograms of aliquots from the anaerobic microsomal incubations clearly show the parent compound I and the single N-oxide II, indicating that microsomal enzymes can catalyse the bioactivation of I in vitro. Interestingly, RIF-1 tumour homogenates contained much higher concentrations of the reduced metabolites II and III than occurred in plasma, suggesting this tumour tissue is capable of reductive bioactivation of I in vivo.

ACKNOWLEDGEMENTS

We thank Dr. Carey Smithen (Roche Products, Welwyn Garden City, U.K.) for Ro 07-0913 and Dr. Bill Lee (Stamford Research International, California, CA, U.S.A.), Dr. Peter Wardman (Gray Laboratory, Northwood, U.K.) and Dr. Tom Strike (U.S. National Cancer Institute, Bethesda, MD, U.S.A.) for supplies of I, II and III.

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