Journal of Chromatography, 563 (1991) 283–298
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5641

Assay of metyrapone, metyrapol and the isomeric mono-N-oxides of metyrapone in biological fluids by high-performance liquid chromatography

JOEL I. USANSKY[a] and LYAQUATALI A. DAMANI[b,*]

Department of Pharmacy, University of Manchester, Manchester M13 9PL (U.K.)

(First received April 3rd, 1990; revised manuscript received September 13th, 1990)

ABSTRACT

A sensitive high-performance liquid chromatographic (HPLC) method for the analysis of metyrapone [2-methyl-1,2-di-(3-pyridyl)-1-propanone], its reduced metabolite metyrapol and metyrapone mono-N-oxide metabolites in biological fluids is reported. These components were extracted into dichloromethane (2 × 5 ml) from alkalinised microsomal incubates, urine and blood (final pH about 12.5), or from cytosolic incubates at pH 7.4 (final aqueous volume 2–4 ml). Recoveries were in the range 70–100% under these conditions. The intact drug and metabolites were separated by reversed-phase HPLC with ultraviolet detection at 261 nm. All calibration curves were linear (correlation coefficient >0.997). For the analysis of hepatic microsomal or cytosolic incubates, the coefficient of variation was less than 10% for samples over the range 2.5–250 nmol/ml N-oxides and 10–250 nmol/ml metyrapol. Measurement of metyrapone and metyrapol in rat blood (0.25-ml sample volume) was linear in the ranges 4.4–265 and 26–263 nmol/ml, respectively, the lower concentration being the limit of detection. The coefficient of variation was less than 20% for samples over the ranges tested for both these compounds. The N-oxide metabolites were not detectable in blood using this assay, their concentrations being below the limit of detection.

INTRODUCTION

Metyrapone [2-methyl-1,2-di-(3-pyridyl)-1-propanone, SU-4885] has been used to assess the adrenocorticotropic hormone secretory capacity of the anterior lobe of the pituitary gland [1,2]. It is also used in the treatment of Cushing's syndrome and occasionally as a diuretic in patients with resistant oedema. Metyrapone inhibits 11β-hydroxylase, a cytochrome P-450-dependent enzyme located in the mitochondria of the adrenal cortex. Metyrapone is also used in drug metabolism studies as an inhibitor of cytochrome P-450 isozymes [3]. Indeed, the inhibition of enzymatic activity by metyrapone, as well as by other inhibitors such

[a] Present address: Upjohn Center for Clinical Pharmacology, University of Michigan, Ann Arbor, MI 48109-0504, U.S.A.

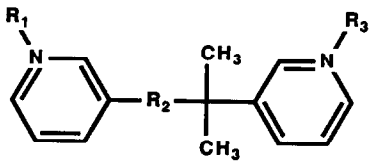
[b] Present address: Chelsea Department of Pharmacy, King's College London, Manresa Road, London SW3 6LX, U.K.

0378-4347/91/$03.50

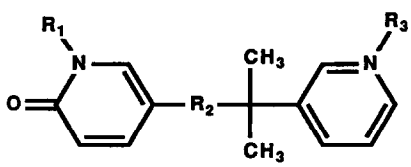
as SKF 525A or carbon monoxide, is a criterion which can be used to establish whether cytochrome P-450 is involved in a biotransformation process [4].

Despite the use of metyrapone in humans as a diagnostic agent and its widespread use in drug metabolism studies, there are few reports describing the pharmacokinetics of metyrapone or the enzymes catalysing its metabolism. The metabolic options open to metyrapone (Fig. 1) are (i) reduction of the keto group to the secondary alcohol metyrapol [5,6], (ii) oxidation at either of the two pyridyl nitrogen atoms yielding two isomeric metyrapone mono-N-oxides [7,8], (iii) α-C-oxidation of metyrapone to an α-pyridone moiety [9], and (iv) N-methylation to yield two isomeric metyrapone mono-N-methylated metabolites [10].

A number of publications detailing analytical methods for the measurement of



Compound	Abbreviation	R_1	R_2	R_3
Metyrapone	MP	—	C = O	—
Metyrapone N-oxide	MP-NO(I)	O	C = O	—
Metyrapone N-oxide	MP-NO(II)	—	C = O	O
Metyrapol	MPOL	—	CH-OH	—
N-Methyl metyrapone	NMe-MP(I)	CH_3	C = O	—
N-Methyl metyrapone	NMe-MP(II)	—	C = O	CH_3



2-Methyl-1-[3-(6-oxopyridyl)]-2-(3-pyridyl)-1-propanone	α-Pyridone	H	C = O	—

Fig. 1. Structures of metyrapone and its *in vitro* metabolites.

metyrapone or metyrapol have previously been reported. A quantitative bioassay for metyrapone-related material in plasma was developed by Sprunt *et al.* [6]; metyrapone was assayed by measuring the ability of the plasma to inhibit hydroxylation of deoxycorticosterone (DOC) following co-incubation of rat adrenal homogenate, NADPH, DOC and [^{14}C]DOC [6]. Radioimmunoassay has been used to measure both metyrapone and metyrapol [11,12]: plasma samples were extracted with dichloromethane, then intact drug and metyrapol were separated on a silica column and quantified by radioimmunoassay using tritiated metyrapone, rabbit and goat anti-rabbit immunoglobulins. In another method metyrapone in plasma was extracted with diethyl ether, cleaned up on a silica column, then reacted with cyanogen bromide and *p*-aminoacetophenone to enable fluorescence detection of the derivatised product [13].

Chromatographic techniques such as paper chromatography [5] and thin-layer chromatography (TLC) [6,14–16] have also been used to separate metyrapol from metyrapone. Quantitation was achieved either by elution from the silica with quantitation using UV absorption [14], or elution followed by derivatisation with cyanogen bromide and *p*-aminosalicylic acid (König reaction), then UV measurement [16]. Gas chromatography (GC) has been used for the analysis of metyrapone and metyrapol from the urine of patients undergoing the metyrapone test [17] or from rat plasma [18]. However, when introduced into the gas chromatograph both of the isomeric metyrapone mono-N-oxides yielded only one peak corresponding to the retention time for metyrapone, thereby indicating catalytic deoxygenation had occurred under GC conditions [8,19].

A qualitative reversed-phase high-performance liquid chromatography (HPLC) method has been used to identify the metabolic products of metyrapone from incubations with rat liver microsomes and cytosol [7,9,19,20]. More recently, a different reversed-phase HPLC system was used to quantitatively assay only metyrapone and metyrapol in liver extracts [21]. An alternative method using a normal-phase HPLC system was used by Dixon and co-workers [22,23] to separate metyrapone from metyrapol and the metyrapone N-oxides in extracts of urine.

We proposed to investigate the *in vivo* metabolism and pharmacokinetics of metyrapone in rats, and to study the *in vitro* metabolism of metyrapone by various organ homogenates. None of the published reports appeared to satisfy our requirements for a sensitive and specific assay method for the intact drug and its keto-reduced, N-oxidised and α-pyridone metabolites because they either lacked specificity, lacked sensitivity or were qualitative methods only. In this paper we describe the development of extraction and analytical methodologies to measure metyrapone, metyrapol amd their isomeric N-oxides by HPLC in biological fluids.

EXPERIMENTAL

Reagents and materials

Metyrapone was a gift from Ciba Labs. (Horsham, U.K.). Sodium borohydride, *m*-chloroperoxybenzoic acid and 2,3-dipyridyl were purchased from Aldrich (Gillingham, U.K.). HPLC-grade acetonitrile was from Rathburn (Walkerburn, U.K.). All other chemicals were from BDH (Poole, U.K.), and all reagents were used as received.

Preparation of metabolites

Metyrapol was synthesised by sodium borohydride reduction of metyrapone using the method of Kraulis *et al.* [5]. The metyrapone mono-N-oxides were synthesised by the action of *m*-chloroperoxybenzoic acid on metyrapone [7]. The desired metyrapone mono-N-oxides were separated by column chromatography, preparative TLC on silica gel GF_{254}, preparative HPLC and finally recrystallisation [24]. The metyrapol mono-N-oxides were prepared by sodium borohydride reduction of the metyrapone mono-N-oxides as outlined by Damani *et al.* [19]. The α-pyridone metabolite of metyrapone was prepared from incubations of metyrapone with either partially purified rabbit liver aldehyde oxidase or rat liver cytosol and purified by preparative TLC [24]. The identity of all synthesised metabolites was confirmed by melting point, low- and high-resolution electron-impact mass spectrometry, NMR, UV and IR spectroscopy. The authentic compounds were dissolved in methanol and stored at −23°C. All compounds were found to be stable under these conditions giving only one peak by HPLC and TLC when checked at intervals.

Column packing

Standard empty HPLC columns (250 mm × 4.6 mm I.D. and 150 mm × 4.6 mm I.D.) fitted with zero dead-volume end pieces and the packing materials (Whatman Partisil 10-μm ODS-1 and Spherisorb 5-μm ODS-1) were obtained from HPLC Technology (Wilmslow, U.K.). The analytical columns were packed using an upward slurry packing technique by suspending the material in isopropyl alcohol and packing with hexane at approximately 500 bar using an air-driven MCP-110 pump (Haskel Engineering, Burbank, CA, U.S.A.). The columns were then conditioned with isopropyl alcohol followed by methanol, and their efficiencies assessed prior to use by injection of phenol solution using a mobile phase of methanol–water (60:40, v/v) at a flow-rate of 1.0 ml/min.

HPLC equipment

The HPLC equipment comprised a M6000A Waters Assoc. pump (Milford, MA, U.S.A.) and a Rheodyne Model 7125 injector (Cotati, CA, U.S.A.) fitted with a 20-μl sample loop. The detectors used were either a Waters 440 fixed-wavelength detector operating at 254 nm or a Pye Unicam LC3 variable-wave-

length detector at 261 nm. The detector output was connected either to a Servoscribe 1S recorder (Smith Industries, London, U.K.) or to a Trilab 2000 integrating computer (Trivector, Sandy, U.K.). A saturator column (100 mm × 4.6 mm. I.D.) filled with 40–60 μm silica particles was placed between the pump and the injection valve in order to presaturate the mobile phase with silicate ions to hinder dissolution of the analytical column.

Preparation of hepatic tissue fractions

Male Sprague–Dawley rats (approximately 150 g) were killed by cervical dislocation, and their livers were excised, weighed and homogenised in phosphate buffer (pH 7.4, 0.2 *M*) using a Potter–Elvejhem homogeniser. After centrifugation at 10 000 *g* for 30 min, the resultant supernatant was ultracentrifuged at 140 000 *g* for 1 h. The sedimented microsomes were "washed" by resuspension in buffer and recentrifugation at 140 000 *g*; the cytosolic fraction was "washed" by recentrifugation at 140 000 *g*.

Optimisation of chromatographic conditions

Phosphate buffer was prepared by dissolving sodium phosphate ($NaH_2PO_4 \cdot 12\ H_2O$) in water, then adjusting to the required pH (5, 6, 7, 7.4 and 8) with either sodium hydroxide or hydrochloric acid as necessary. The mobile phase was prepared by mixing appropriate proportions of acetonitrile and phosphate buffer by volume, followed by degassing under vacuum. Using a 10-μm Partisil ODS-1 column (250 mm × 4.6 mm I.D.) and various combinations of acetonitrile and phosphate buffer, the pH and molar concentration of the mobile phase buffer, the concentration of the organic solvent in the mobile phase and eluent flow-rate were altered in turn to assess their effects on the retention times and peak widths of each component.

Recovery from biological samples

The extraction recovery of metyrapol and the two metyrapone N-oxides was assessed by preparing a simulated incubation mixture as follows: a 25-ml incubation flask containing 0.5 ml of metyrapone solution (10 m*M* in 0.2 *M* phosphate buffer), 0.5 ml of phosphate buffer (pH 7.4, 0.2 *M*; to simulate cofactors), 0.5 ml of sodium hydroxide (1.0 *M*; to terminate enzyme activity, final pH approximately 12.5) and 1.0 ml of microsomal preparation (equivalent to 0.5 g wet weight of liver) was spiked with 250 nmol of metyrapol and MP-NO(II) and with 50 nmol of MP-NO(I). Preliminary experiments indicated that these quantities of metabolites would be produced following incubation with the partially purified enzymes. After vortex-mixing, 0.5 ml of 2,3′-dipyridyl solution (0.5 m*M*; internal standard) and 1.0 g sodium chloride were added, vortex-mixed for 15 s, transferred to a glass extraction tube, and then extracted and analysed as described below for microsomes.

The procedure for the recovery from cytosol was similar to that of microsomes

except that (i) 0.5 ml of cytosol (equivalent to 0.5 g wet weight of liver) was substituted for microsomes, (ii) the flask was spiked with 250 nmol of metyrapol and the α-pyridone metabolite, and (iii) 0.5 ml of hydrochloric acid (1.0 *M*; to neutralise the alkali) was also added.

The extraction recovery from blood was assessed by spiking an extraction tube containing 0.25 ml of control rat blood, 0.5 ml of sodium hydroxide (1.0 *M*) and 0.1 ml of internal standard solution (0.5 m*M* in water) with 55.3 nmol of metyrapone and 54.8 nmol of metyrapol (final pH approximately 12.2). Recovery samples were extracted and analysed as for blood specimens (see section below).

The extraction recovery from urine was checked by spiking extraction tubes with 0.5 ml of control rat urine, 0.5 ml of sodium hydroxide (1.0 *M*) and 0.1 ml of internal standard solution (500 nmol in water) with 55.3 nmol of metyrapone and 54.8 nmol of metyrapol (final pH approximately 12.5). Recovery samples were extracted and analysed as for blood specimens (see section below).

Precision and accuracy of assays

Simulated incubation mixtures and control rat blood and urine samples were prepared as above and spiked with intact drug and metabolites as appropriate in triplicate. The spiked samples were then processed as described below for each biological medium.

Assay of metyrapol and metyrapone N-oxides in microsomal incubates

A typical incubation mixture comprised rat hepatic microsomes (equivalent to 0.5 g wet weight of liver), $NADP^+$ (2 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1.0 U), and magnesium chloride (42 μmol) as co-factors and 5.0 μmol of metyrapone as substrate in a total volume of 2.0 ml of phosphate buffer (pH 7.4, 0.2 *M*). Sodium hydroxide (0.5 ml, 1.0 *M*) was added to terminate enzymatic activity. Following addition of the internal standard (250 nmol of 2,3′-dipyridyl in 0.5 ml of water) the reaction mixture was transferred to a screw-capped glass extraction tube containing 1.0 g of sodium chloride and then extracted twice with 5-ml portions of dichloromethane. The combined organic layers were collected in a tapered glass evaporating tube, concentrated to dryness in a water bath at 40°C and reconstituted in 25 μl of methanol for injection onto the HPLC column. The chromatographic system consisted of a 10-μm Partisil ODS-1 column (250 mm) using acetonitrile–phosphate buffer (0.067 *M*, pH 7.4) in the proportion 20:80 (v/v) as mobile phase at ambient temperature and at a flow-rate of 2.0 ml/min. For calibration, solutions of metabolites were added to incubation flasks to give concentrations in the range 2.5–125 nmol/ml MP-NO(I), 2.5–250 nmol/ml MP-NO(II) and 5–250 nmol/ml metyrapol.

Assay of metyrapol and the α-pyridone in cytosolic incubates

The procedure for the analysis of cytosolic metabolites was identical to that of microsomes except that (i) rat hepatic cytosol (equivalent to 0.5 g wet weight of

liver) was substituted for the microsomal suspension, and (ii) hydrochloric acid (0.5 ml, 1.0 *M*) was added to the mixture prior to extraction with dichloromethane to neutralise the sodium hydroxide. Calibration samples were spiked with 5–125 nmol/ml α-pyridone and 5–250 nmol/ml metyrapol.

Assay of metyrapone and metyrapol in blood

Aliquots of blood (0.25 ml) from rats administered metyrapone were sampled at the appropriate times and placed into tubes containing alkali (0.5 ml, 1.0 *M* sodium hydroxide). Following addition of the internal standard (0.1 ml of 2,3′-dipyridyl containing 50 nmol) the mixture was extracted twice with 5-ml portions of dichloromethane and the organic phase evaporated and reconstituted as for microsomes. The HPLC system comprised a 5-μm Spherisorb ODS-1 column (150 mm) using a mobile phase of acetonitrile–phosphate buffer (0.067 *M*, pH 7.4) in the proportion 25:75 (v/v). Calibration samples were spiked with 4.4–265 nmol/ml metyrapone and 26.3–263 nmol/ml metyrapol.

Assay procedure for urinary metabolites of metyrapone

Aliquots of urine (0.5 ml) from rats administered metyrapone were placed into glass extraction tubes to which alkali (0.5 ml, 1.0 *M* sodium hydroxide) had previously been added. Following addition of the internal standard (2,3′-dipyridyl, 1.0 ml containing 500 nmol) and 1.0 g of sodium chloride, the mixture was rapidly extracted with two 5-ml portions of dichloromethane and the combined organic phase evaporated and reconstituted as for microsomes. The HPLC system comprised a 10-μm Partisil ODS-1 column (250 mm) using a mobile phase of acetonitrile–phosphate buffer (0.01 *M*, pH 7.4) in the proportion 15:85 (v/v) at 3.0 ml/min. Calibration samples were spiked with up to 500 nmol of metyrapone N-oxides, 1500 nmol of metyrapol N-oxides and 200 nmol of metyrapol.

Calculations

Peak-height ratios of the test compound to internal standard were plotted against known concentrations for each component; at least five to eight points were used to construct a calibration line. Values for unknowns were calculated from the slope and intercept by linear regression of the calibration curves.

RESULTS AND DISCUSSION

Optimisation of chromatographic conditions

The qualitative HPLC system used by Damani *et al.* [20] was used as a starting point to determine the optimum conditions for the separation of all the metabolites. Using simulated incubation mixtures various chromatographic parameters were altered to try to improve the separation between the overlapping N-oxides and the α-pyridone metabolites. When the pH of the mobile phase was adjusted between 5.0 and 8.0, the retention times for all compounds were longest at pH 6.0

TABLE I

INFLUENCE OF ELUENT pH ON THE CHROMATOGRAPHIC RETENTION TIMES OF METYRAPONE, ITS *IN VITRO* METABOLITES AND THE INTERNAL STANDARD (2,3'-DIPYRIDYL)

Column: Partisil 10 ODS-1 (250 mm × 4.6 mm I.D.); eluent: acetonitrile–0.067 *M* phosphate buffer, 25:75 (v/v); flow-rate: 2.5 ml/min.

Compound	Retention time[a] (min)				
	pH 5.0	pH 6.0	pH 7.0	pH 7.4	pH 8.0
Metyrapone	10.8	12.0	10.8	10.7	9.7
MP-NO(I)	6.1	6.5	5.9	5.9	5.2
MP-NO(II)	5.0	5.3	4.9	4.8	4.3
Metyrapol	7.2	9.8	8.8	8.7	7.7
α-Pyridone	5.2	5.2	4.8	4.7	4.4
2,3'-Dipyridyl	7.0	7.8	7.2	7.2	6.5

[a] Retention time of unretained peak = 1.2 min.

and minimal at pH 8.0 (Table I); however, the separation selectivity was not enhanced and the compounds were eluted in the same order. Increasing the molarity of the phosphate buffer from zero (*i.e.* no buffer) to 0.133 *M* had no discernible effect on the retention times with the exception of the α-pyridone metabolite whose retention time decreased in the absence of buffer. This variability may have been due to the uncontrolled pH of the water used to prepare the mobile phase. The effect of alterations in the proportion of acetonitrile in the mobile phase and the effect of flow-rate did not enhance the separation of the overlapping components. Proportions of acetonitrile greater than 25% caused the two mono-N-oxide peaks to partially merge together; the optimum acetonitrile content was approximately 20–25%. Separation selectivity can frequently be achieved by replacing the organic modifier with methanol or tetrahydrofuran, but these solvents produced only poorly defined peak shapes with tailing.

Thus, it was not possible to separate the mono-N-oxides from the α-pyridone metabolite by altering the HPLC conditions as described above. Fortunately, for the assay of the *in vitro* incubations, these overlapping metabolites are generated by different subcellular fractions, the mono-N-oxides and the α-C-oxidised compound being formed by the microsomal and cytosolic fractions, respectively. Therefore analysis of incubation mixtures is still possible providing the enzyme preparations are "washed" so that there is no contamination of the microsomal or cytosolic enzymes by each other.

Figs. 2–5 illustrate typical chromatograms of metyrapone and its metabolites derived from extracts of incubations of metyrapone with either hepatic microsomal preparations (Fig. 2), hepatic cytosolic preparations (Fig. 3), or from extracts of blood (Fig. 4) or urine (Fig. 5) samples following a dose of metyrapone to rats. Control samples were free from interference at the retention times of interest.

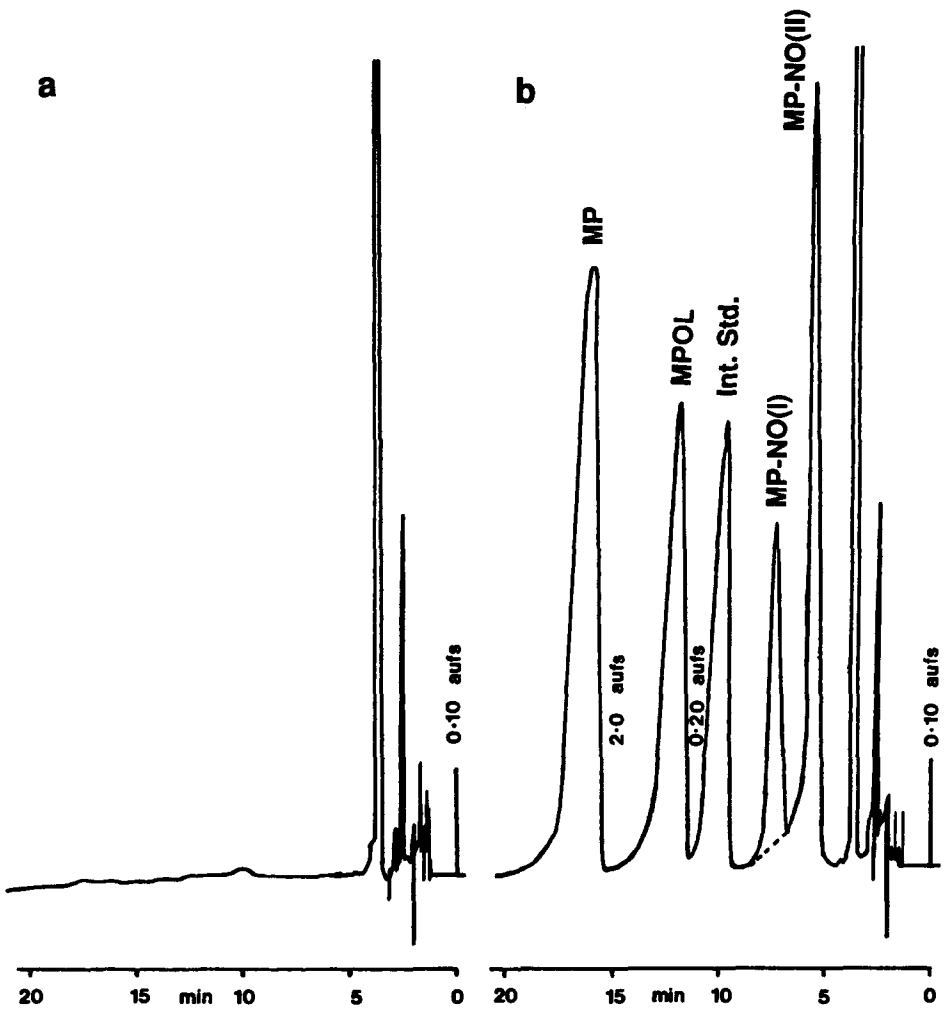


Fig. 2. Typical chromatograms for extracts of (a) control rat hepatic microsomal incubation and (b) incubation mixture of metyrapone with rat hepatic microsomes using 2,3′-dipyridyl as internal standard. For peak identification, see Fig. 1.

Recovery from biological samples

Having developed optimal separation, the chromatographic parameters were then used to assess the extraction recovery and to develop the assay procedure. In order to maximise the sensitivity of the HPLC assay the UV absorption characteristics of metyrapone, its reduced and N-oxide metabolites and the internal standard were assessed in the assay HPLC mobile phase. Table II shows that all the metyrapone derivatives except the α-pyridone absorb maximally around 260 nm. At this wavelength all the compounds have a similar molar absorptivity.

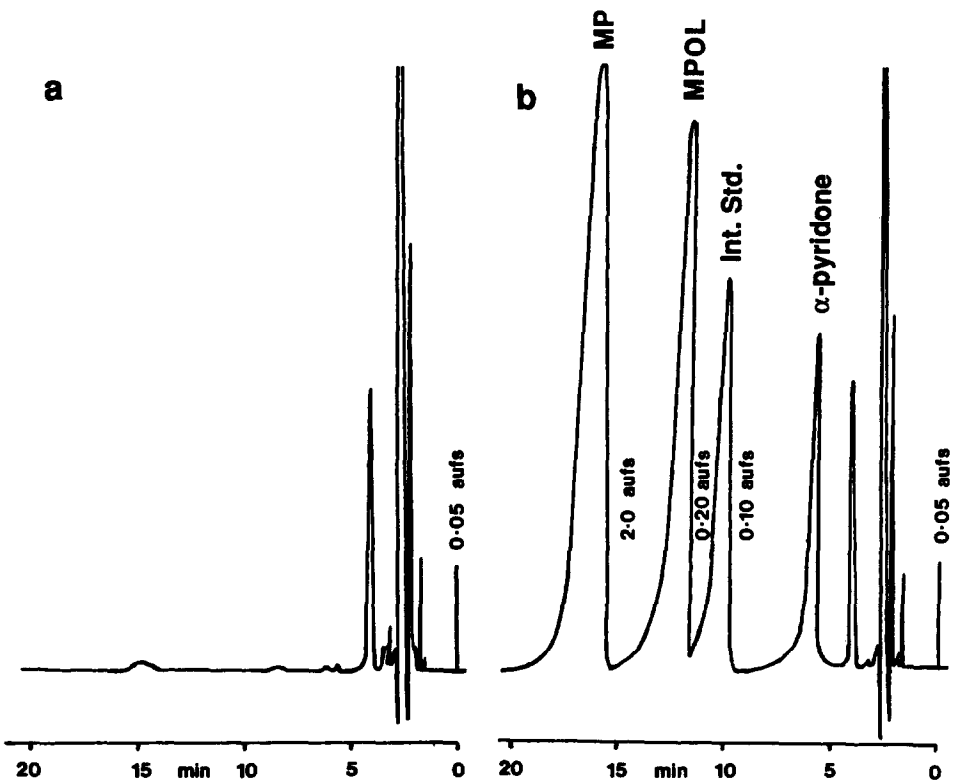


Fig. 3. Typical chromatograms for extracts of (a) control rat hepatic cytosolic incubation and (b) incubation mixture of metyrapone with rat hepatic cytosol using 2,3′-dipyridyl as internal standard. For peak identification, see Fig. 1.

The extraction recovery of the metabolites are reported in Table III. The extraction of metyrapol from simulated incubation mixtures was 97% from microsomes and 95% from cytosol. The extraction of MP-NO(I) was quantitative, but was only about 70% for MP-NO(II). Although N-oxide-containing compounds are polar and are generally not well extracted by organic solvents the metyrapone N-oxides extract reasonably efficiently, probably due to the lipophilic nature of the remainder of the molecule. The reason for the relatively large differences in extraction efficiency between the two N-oxides is unknown but may be due to the differences in basic strengths of the non-oxidised pyridine moieties. The extractability of the α-pyridone under the same basic conditions as for microsomes resulted in only 22% recovery. This could be due to ionisation of the amphoteric α-pyridone moiety; in contrast the extraction under neutral conditions was essentially quantitative. For all metabolites, the extraction efficiency was minimally affected by the lack of addition of sodium chloride, protein or by a second extraction step.

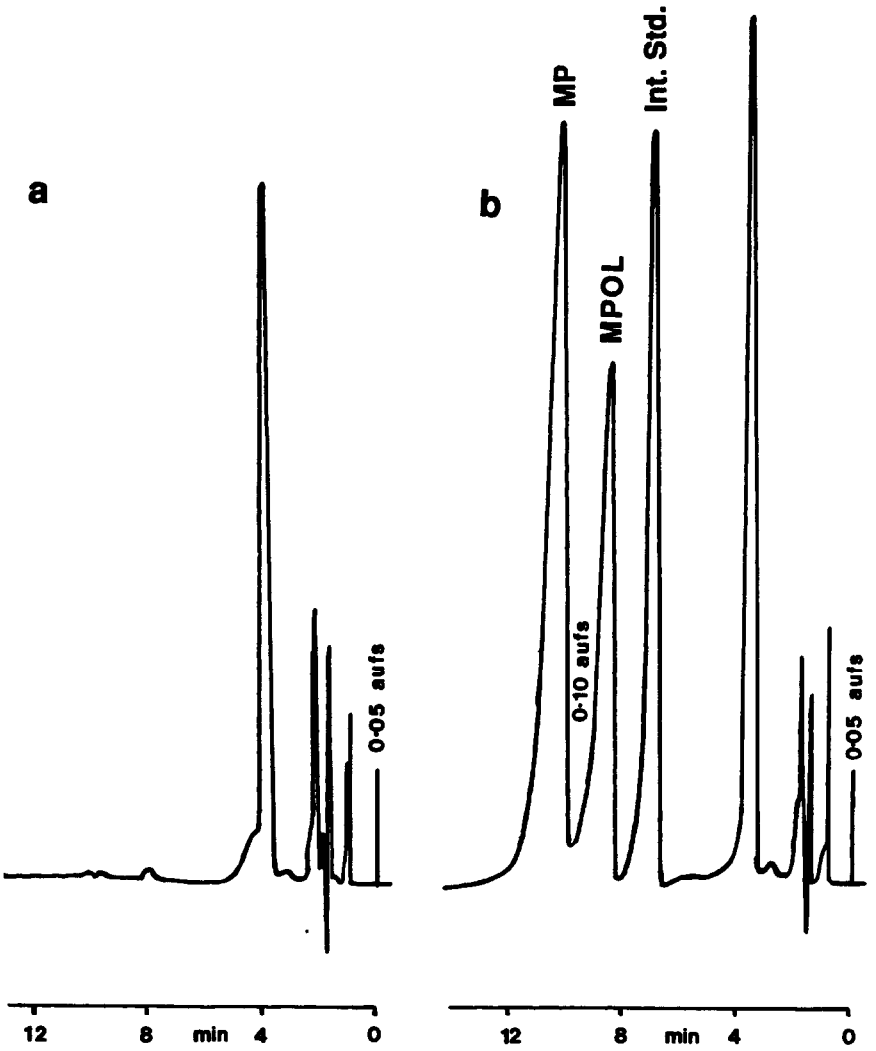


Fig. 4. Representative chromatograms for extracts of (a) pre-dose rat blood and (b) blood following administration of 50 mg/kg metyrapone to rats. For peak identification, see Fig. 1.

Precision and accuracy of assays

The calibration curves for the assay of *in vitro* incubations were linear and reproducible in the ranges 2.5–125, 2.5–250 and 5–250 nmol/ml incubate for MP-NO(I) and MP-NO(II) and metyrapol, respectively. The precision of the microsomal assay, as measured by the coefficient of variation (C.V.), was less than 10% for both of the metyrapone N-oxides over this range and for metyrapol at 10 nmol/ml or above, and less than 20% at 5 nmol/ml (Table IV). The accu-

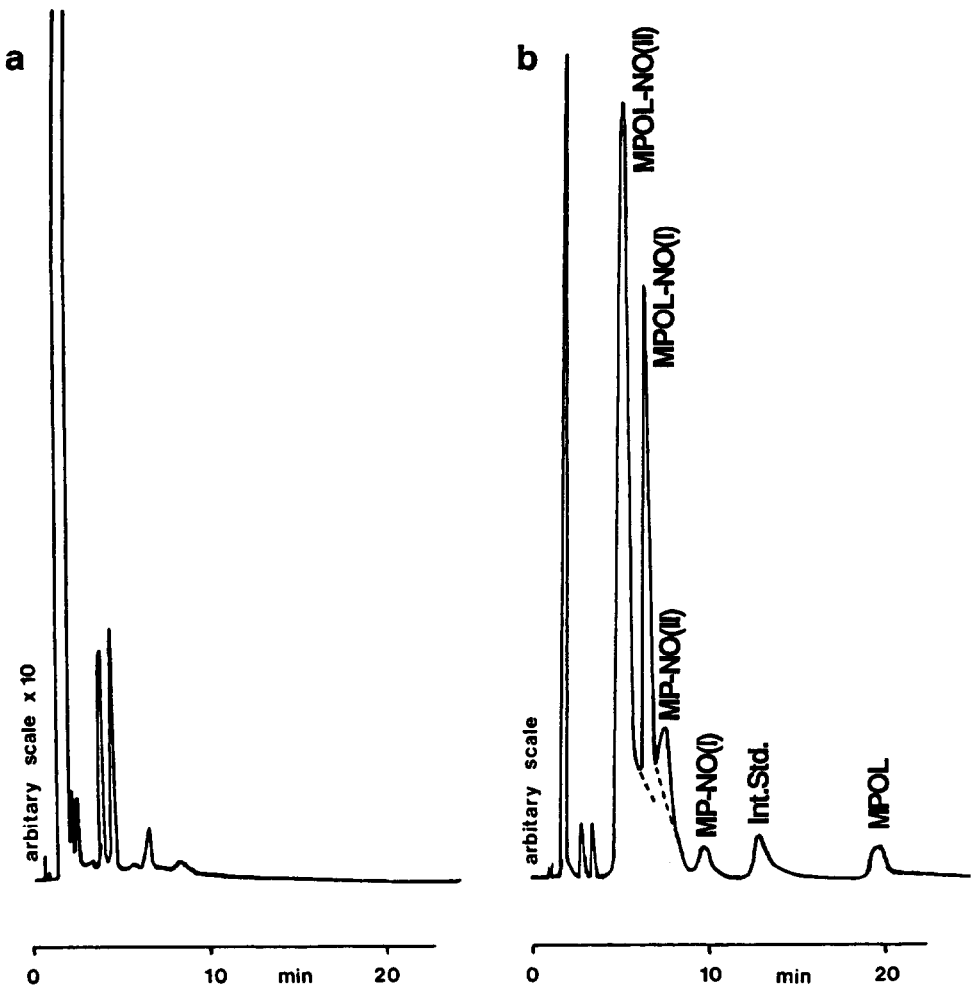


Fig. 5. Representative chromatograms for extracts of (a) control rat urine and (b) urine following administration of 50 mg/kg metyrapone to rats. For peak identification, see Fig. 1.

racy, as measured by the mean percentage difference from the spiked concentration (M.D.), was less than 10% for all three compounds. The limit of detection, defined as the lowest concentration with C.V. and M.D. values less than 10% was 2.5 nmol/ml for the N-oxides and 10 nmol/ml for metyrapol. For the cytosolic assay, control samples did not interfere with the assay and the calibration curves were linear and reproducible. Regression analysis of the data gave a correlation coefficient of not less than 0.996 for each component. The inter-day assay reproducibility was satisfactory as judged by similar C.V. and M.D. values on two other days.

TABLE II

UV ABSORPTION CHARACTERISTICS OF METYRAPONE, ITS METABOLITES AND THE INTERNAL STANDARD IN THE HPLC ELUENT USED FOR THE ANALYSIS OF THE MICROSOMAL AND CYTOSOLIC INCUBATIONS

Eluent composition: acetonitrile–0.067 *M* phosphate buffer, pH 7.4, 20:80 (v/v).

Compound	λ_{max} (nm)	Log molar absorptivity (ε)
Metyrapone	264	3.76
MP-NO (I)	265	4.04
MP-NO (II)	263	4.14
Metyrapol	261	3.85
α-Pyridone	283	3.94
2,3'-Dipyridyl	275	4.05

For analysis of blood and urine, pre-dose control samples did not exhibit interference at the retention times of interest (Figs. 4 and 5). Calibration curves for all components were linear and reproducible. The precision and accuracy of the blood assay was good with C.V. and M.D. values not greater than 20% for both metyrapone and metyrapol at all concentrations above 4.4 and 26.5 nmol/ml for metyrapone and metyrapol, respectively (Table V). The limit of detection for the blood assay was set at the above concentrations. Analysis of extracts of urine samples showed the presence of large amounts of the mono-N-oxide metabolites of metyrapone and metyrapol, small amounts of metyrapol and the absence

TABLE III

EXTRACTION RECOVERY OF METYRAPONE AND ITS METABOLITES FROM BIOLOGICAL MEDIA

Biological medium	Compound	Recovery (mean ± S.D.) (%)	C.V. (%)	*n*
Microsomes	MP-NO(I)	100.0 ± 4.7	4.7	4
	MP-NO(II)	68.8 ± 4.6	6.7	4
	Metyrapol	97.3 ± 2.4	2.4	4
Cytosol	α-Pyridone	98.5 ± 2.1	2.2	2
	Metyrapol	95.0 ± 1.4	1.5	2
Blood	Metyrapone	95.4 ± 13.6	14.2	2
	Metyrapol	93.2 ± 4.4	4.8	2
Urine	Metyrapone	93.1 ± 11.3	8.2	3
	Metyrapol	89.3 ± 8.6	6.5	3
	MP-NO(I)	95.3 ± 9.5	4.5	3
	MP-NO(II)	72.5 ± 5.5	7.8	3

TABLE IV

PRECISION AND ACCURACY OF THE HPLC ASSAY FOR THE MICROSOMAL METABOLITES OF METYRAPONE

Compound	Spiked concentration (nmol/ml)	Concentration found (mean ± S.D.) (nmol/ml)	C.V. (%)	M.D.[a] (%)	n^b
Metyrapol	250	251 ± 4.74	1.89	0.4	3
	25	23.2 ± 0.928	4.00	−7.2	3
	10	9.85 ± 0.263	2.67	−1.5	3
	5	5.19 ± 0.875	16.9	3.8	2
MP-NO(I)	125	126 ± 3.30	2.62	0.8	3
	12.5	12.3 ± 0.130	1.06	−1.6	3
	5	4.95 ± 0.218	4.40	−1.0	3
	2.5	2.58 ± 0.118	4.57	3.2	3
MP-NO(II)	250	252 ± 5.23	2.08	0.8	3
	25	24.8 ± 0.425	1.71	−0.8	3
	10	9.93 ± 0.385	3.88	−0.7	3
	5	5.22 ± 0.263	5.04	4.4	3
	2.5	2.73 ± 0.055	2.01	9.2	2

[a] Mean percentage difference, M.D. = (mean − spiked)/spiked × 100%.
[b] Performed in triplicate on two separate days.

TABLE V

PRECISION AND ACCURACY OF THE HPLC ASSAY FOR METYRAPONE AND METYRAPOL IN BLOOD

Compound	Spiked concentration (nmol/ml)	Concentration found Mean ± S.D. (nmol/ml)	C.V. (%)	M.D.[a] (%)	n
Metyrapol	263	281 ± 27.6	9.81	6.3	3
	87.7	86.8 ± 7.85	9.04	−1.0	3
	43.9	46.1 ± 3.55	7.71	5.0	3
	26.3	24.6 ± 4.56	18.6	6.7	3
Metyrapone	265	285 ± 27.0	9.44	7.5	3
	88.5	86.7 ± 9.12	10.5	−2.0	3
	44.3	50.4 ± 2.04	4.0	14.0	3
	26.6	25.7 ± 1.86	7.24	−3.2	3
	8.85	10.6 ± 1.06	10.0	20.0	3
	4.42	3.67 ± 0.33	8.92	−17.0	3

[a] Mean percentage difference, M.D. = (mean − spiked)/spiked × 100%.

of metyrapone. For the urine assay the major problem was the lack of complete separation of all compounds present. The best separation possible was obtained using a lower proportion of acetonitrile together with a higher flow-rate. It was not possible to quantify the α-pyridone metabolite in urine due to overlap with other metabolites.

Recently, metyrapone has been shown to be N-methylated *in vitro* to two isomeric quaternised products by two homogeneous amine N-methyltransferases. These enzymes were isolated from rabbit liver and required the presence of S-adenosylmethionine as methyl group donor [10]. The related compound, pyridine, also undergoes N-methylation to yield a quaternary amine derivative [25]. Since these compounds bear an ionised quaternary amine group they would be extremely polar and would not be expected to partition into the extraction solvent used in this investigation (dichloromethane). Additionally we anticipate that these ionised compounds would, if formed, elute as unretained components on the HPLC systems used. We saw no evidence for the presence of these metabolites.

In conclusion, we have developed quantitative HPLC assays for the analysis of metyrapone and its reduced and N-oxidised metabolites in incubation media, and, with some minor modifications, also developed quantitative assays for these compounds in blood and urine.

ACKNOWLEDGEMENTS

J.I.U. gratefully acknowledges support by a grant from the Science and Engineering Research Council, U.K.

REFERENCES

1 G. W. Liddle, H. L. Estep, J. W. Kendall, W. C. Williams and A. W. Townes, *J. Clin. Endocrinol. Metab.*, 19 (1959) 875.
2 E. M. Gold, R. Bigler, S. Newman, M. Angers and V. C. DiRaimondo, *Clin. Res.*, 7 (1959) 64.
3 B. Testa and P. Jenner, *Drug Metab. Rev.*, 12 (1981) 1.
4 P. G. Wislocki, G. T. Miwa and A. Y. H. Lu, in W. B. Jakoby (Editor), *Enzymatic Basis of Detoxication*, Academic Press, New York, 1980, p. 135.
5 I. Kraulis, H. Traikov, M. P. Li, C. P. Lantos and M. K. Birmingham, *Can. J. Biochem.*, 46 (1968) 463.
6 J. G. Sprunt, M. C. K. Browning and D. M. Hannah, *Mem. Soc. Endocrinol.*, 17 (1968) 193.
7 P. A. Crooks, L. A. Damani and D. A. Cowan, *J. Pharm. Pharmacol.*, 33 (1981) 309.
8 J. De Graeve, J. E. Gielen, G. F. Kahl, K. H. Tuttenberg, R. Kahl and B. Maume, *Drug Metab. Dispos.*, 7 (1979) 166.
9 L. A. Damani, P. A. Crooks and D. A. Cowan, *Drug Metab. Dispos.*, 9 (1981) 270.
10 P. A. Crooks, C. S. Godin, L. A. Damani, S. S. Ansher and W. B. Jakoby, *Biochem. Pharmacol.*, 37 (1988) 1673.
11 A. W. Meikle, S. C. West, J. A. Weed and F. H. Tyler, *J. Clin. Endocrinol. Metab.*, 40 (1975) 290.
12 A. W. Meikle, *Methods Enzymol.*, 84 (1982) 585.
13 A. W. Meikle, W. Jubiz, C. D. West and F. H. Tyler, *J. Lab. Clin. Med.*, 74 (1969) 515.
14 G. F. Kahl, *Naunyn-Schmiedebergs Arch. Pharmakol.*, 266 (1970) 61.

15 W. Dünges and G. F. Kahl, *Naunyn-Schmiedebergs Arch. Pharmakol.*, 267 (1970) 293.
16 S. Szeberenyi, K. S. Szalay and M. T. Tacconi, *J. Chromatogr.*, 40 (1969) 417.
17 D. M. Hannah and J. G. Sprunt, *J. Pharm. Pharmacol.*, 21 (1969) 877.
18 T. R. Hollands and W. J. Johnson, *Biochem. Med.*, 7 (1973) 288.
19 L. A. Damani, P. A. Crooks and D. A. Cowan, *Biomed. Mass Spectrom.*, 8 (1981) 270.
20 L. A. Damani, P. A. Crooks and J. W. Gorrod, *J. Pharm. Pharmacol.*, 31 (1979) 94P.
21 E. Maser and W. Legrum, *Naunyn-Schmiedebergs Arch. Pharmakol.*, 331 (1985) 283.
22 P. A. F. Dixon, S. E. Okereke and M. C. N. Enwelum, *Comp. Biochem. Physiol.*, 81C (1985) 241.
23 P. A. F. Dixon, C. A. Joseph, S. E. Okereke and M. C. N. Enwelum, in J. W. Gorrod and L. A. Damani (Editors), *Biological Oxidation of Nitrogen in Organic Molecules,* VCH/Ellis Horwood, Weinheim/Chichester, 1985, p. 236.
24 J. I. Usansky, *Ph.D. Thesis,* University of Manchester, Manchester, 1988.
25 M. S. Shaker, P. A. Crooks and L. A. Damani, *J. Chromatogr.*, 237 (1982) 489.