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Determination of isomeric N-oxide metabolites of some substituted 2,4-diaminopyrimidines by reversed-phase ion-pair high-performance liquid chromatography

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ABSTRACT

Reversed-phase ion-pair high-performance liquid chromatography has been investigated for the separation, detection, identification and quantitation of the isomeric 1N- and 3N-oxide metabolites of metoprine, pyrimethamine and trimethoprim. A rapid and sensitive analytical method for the simultaneous determination of the isomeric 1N- and 3N-oxides of metoprine and pyrimethamine was devised for *in vitro* metabolic studies by optimisation of mobile phase pH, pairing-ion concentration, secondary-ion concentration and percentage organic modifier.

INTRODUCTION

Metoprine (MET), pyrimethamine (PYRM) and trimethoprim (TMP) are inhibitors of dihydrofolate reductase. The antibacterial properties of TMP, either alone or in combination with sulphonamides, has been used in the treatment of many common infections such as urinogenital and respiratory tract infections [1]. PYRM exhibits antimalarial activity and has been used for many years [2], whilst MET is a potent inhibitor of human dihydrofolate reductase [3], whose favourable physicochemical properties has indicated its use in the treatment of neoplastic diseases involving the central nervous system [4].

Metabolic studies have indicated that the 1- and 3N-oxides of all three compounds are produced *in vivo* in several species, including man [5–8]. Information regarding the enzymology of these processes is lacking as are quantitative and pharmacokinetic data. A simple analytical method for the simultaneous determination of the isomeric 1N- and 3N-oxides of MET, PYRM and TMP was required for further metabolic studies. A review of the literature indicated numerous analytical methods were available for the parent compounds. Autoradiography, fluorescence-quenching spectrofluorometry and liquid scintillation counting have all been used to determine the N-oxide metabolites of these compounds [5,9–11]. Isomeric TMP N-oxides have been analysed by differential pulse polarography [12], but required chromatographic separation from unchanged TMP by thin-layer chromatography prior to their determination [6]. These methods required either radiolabelled compounds and/or time-consuming sample preparation procedures prior to analysis; therefore alternative methods were sought.

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Direct gas chromatographic determination of the N-oxide metabolites would be inappropriate due to their thermal instability [13]. A high-performance liquid chromatographic (HPLC) method has recently been reported for PYRM and its 3N-oxide metabolite in biological fluids [7], however, no mention of the 1N-oxide was made. Studies in our laboratory using the HPLC method of Coleman *et al.* [7] for PYRM and its 3N-oxide resolved the 1N- and 3N-oxides of MET and PYRM, but lacked selectivity as the 1N-oxides co-eluted with the parent compounds. Low capacity factors were observed with this system for all parent compounds and their N-oxides, *i.e.* 5.8 and 6.0 for PYRM and MET, respectively, with TMP eluting close to the solvent front. Alterations in mobile phase composition and increased octanesulphonic acid ion-pair concentration failed to improve selectivity. Consequently, this ion-pairing agent was considered inappropriate for the requirements of this HPLC application.

Many reports have described the use of sodium dioctylsulphosuccinate as an ion-pairing agent for reversed-phase HPLC. Determinations of amine ingredients in cough-cold liquid [14–16], isoniazid–pyridoxine hydrochloride mixtures [17] and niacin–niacinamide [18] in multivitamin preparations have been accomplished using dioctylsulphosuccinate ion pairs. Minoxidil (2,4-diamino-6-piperidinopyrimidine-3N-oxide) and related compounds have been analysed by similar ion-pair methods [19]. Structural similarities with our 2,4-diaminopyrimidine-N-oxides prompted an investigation into the use of sodium dioctylsulphosuccinate in developing an analytical system for the isomeric 1N- and 3N-oxides of MET, PYRM and TMP.

EXPERIMENTAL

Materials

Metoprine [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine, lot 2282-160-A], pyrimethamine [2,4-diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine, lot 05331] and trimethoprim [2,4-diamino-5-(3′,4′,5′-trimethoxybenzyl)-pyrimidine, lot 06538] were gifts from Wellcome UK (Beckenham, UK). Synthetic N-oxides of MET, PYRM and TMP were prepared by *m*-chlorperbenzoic acid oxidation using the procedure of Craig and Purushotaman [20]. Reagent-grade sodium dioctylsulphosuccinate (99% pure), ammonium nitrate and sodium nitrate were obtained from British Drug Houses (Poole, UK). Laboratory-grade ethyl acetate was supplied by May and Baker (Dagenham, UK). Water was doubly distilled in glass before use. Reagent-grade sodium chloride and perchloric acid (69–71%) were obtained from Fisons (Loughborough, UK). Acetonitrile 230, HPLC grade, was supplied by Koch-Light (Haverhill, UK).

High-performance liquid chromatography

The liquid chromatograph consisted of a Gilson Model 302 pump coupled to a Gilson Model 802 manometric module and a Rheodyne 7125 syringe loading sample injector valve fitted with a 20-μl sample loop. Detection was by means of a variable-wavelength Pye-Unicam LC–UV detector set at 254 nm and a Heathkit chart recorder. The column was a 250 mm × 4.6 mm I.D. seamless tube packed with μBondapak C_{18} obtained from Millipore UK (Harrow, UK), 10 μm particle size. A 50 mm × 2.1 mm I.D. guard column (Jones Chromatography, Llanbradach, UK) packed with Co:Pell ODS, 40 μm average particle size (Technicol, Stockport, UK), was placed immediately before the analytical column.

Solvents were degassed by a 15-min purge with helium and filtered through GF/F glass fibre filter paper (Whatman, Maidstone, UK) after mixing, followed by a further 5-min purge with helium. The optimised mobile phase consisted of acetonitrile–water (52:48, v/v) containing 0.026 *M* sodium dioctylsulphosuccinate as ion-pairing agent and 0.02 *M* ammonium nitrate as secondary ion buffered to pH 3.1 with perchloric acid flowing at 2 ml/min, 105 MPa. After use, the column was routinely washed with 50-ml volumes of acetonitrile–water (52:48, v/v), water and finally acetonitrile.

Optimisation of mobile phase

Optimisation of mobile phase was required to achieve resolution between parent compounds and their N-oxides whilst maintaining good peak symmetry and reasonable analysis time. This was accomplished by investigating the effect of pairing-ion concentration, secondary-ion concentration, percentage organic modifier and mobile phase apparent pH, on retention characteristics. Capacity factors, k, were determined using the equation: $k = (V_R - V_M)/V_M$, where V_R is the retention volume of the analyte and V_M is the retention volume of unretained analyte, estimated by aqueous sodium nitrate (20 mg/ml).

Sample treatment procedures

Authentic reference N-oxides were dissolved in methanol and added to typical microsomal incubates (3 ml) to give mixtures containing between 3 and 450 nmol of reference compound. The internal standard (0.5 mg/ml TMP in 30 μl of 1% lactic acid for PYRM and 0.125 mg/ml in 30 μl of 1% lactic acid for MET) was added and the contents of the flasks quantitatively transferred to screw-capped tubes (10 ml, Sterilin) containing 1 g of sodium chloride. Ethyl acetate (5 ml) was added, and the tubes were capped and extracted on a rocking device for 5 min. The tubes were centrifuged at 2000 *g* to break up emulsions, the upper organic layers were transferred to clean tubes and evaporated to dryness at 45°C under reduced pressure using a Buchler vortex evaporator. Two further extracts with ethyl acetate were concentrated as above, the combined residues dissolved in approximately 100 μl of methanol, and 20-μl aliquots injected onto the column. Peak-height ratios of the authentic N-oxides to internal standard were plotted against concentration.

RESULTS AND DISCUSSION

Initial results of the chromatographic separation of the 2,4-diaminopyrimidines and their N-oxides using sodium dioctylsulphosuccinate as ion-pairing agent were encouraging and are shown in Table I. TMP and its isomeric 1N- and 3N-oxides eluted early and the normal-phase HPLC method described previously [8] for these compounds was considered a more appropriate analytical method. TMP proved useful as an internal standard for PYRM and MET N-oxides. The isomeric N-oxides of PYRM and MET were successfully resolved (Table I) but eluted as fairly broad peaks. Substitution of acetonitrile for methanol as an organic modifier was investigated to improve peak symmetry and resolution.

TABLE I

SEPARATION OF 2,4-DIAMINOPYRIMIDINES AND ISOMERIC 1N- AND 3N-OXIDES USING SODIUM DIOCTYLSULPHOSUCCINATE AS ION-PAIRING REAGENT

Compound	Retention time (min)	
	75% Methanol[a]	70% Methanol[a]
Trimethoprim (TMP)	3.0	5.0
TMP 1N-oxide	2.6	4.7
TMP 3N-oxide	1.8	2.2
Pyrimethamine (PYRM)	5.3	11.5
PYRM 1N-oxide	4.8	10.0
PYRM 3N-oxide	3.9	6.8
Metoprine (MET)	6.4	12.9
MET 1N-oxide	5.5	11.2
MET 3N-oxide	4.0	6.4

[a] Mobile phase: 70 and 75% methanol–water mixtures containing 6.6 m*M* sodium dioctylsulphosuccinate pH 3 (pH adjusted with perchloric acid); flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm).

The effect of incorporating different percentages of acetonitrile in the mobile phase on retention is shown in Fig. 1. At 60% acetonitrile, resolution between the isomeric N-oxides and parent compounds was evident. Reducing the acetonitrile content to 56% led to increased resolution with near baseline separation. However, under these conditions the 1N-oxide peaks were broad with the 3N-oxides peaks showing evidence of tailing. At 52% acetonitrile content, resolution was further improved, but at the expense of peak symmetry. Attempts were made to optimise the mobile phase to improve peak symmetry.

Several approaches have been described to improve peak symmetry in ion-pair HPLC systems.

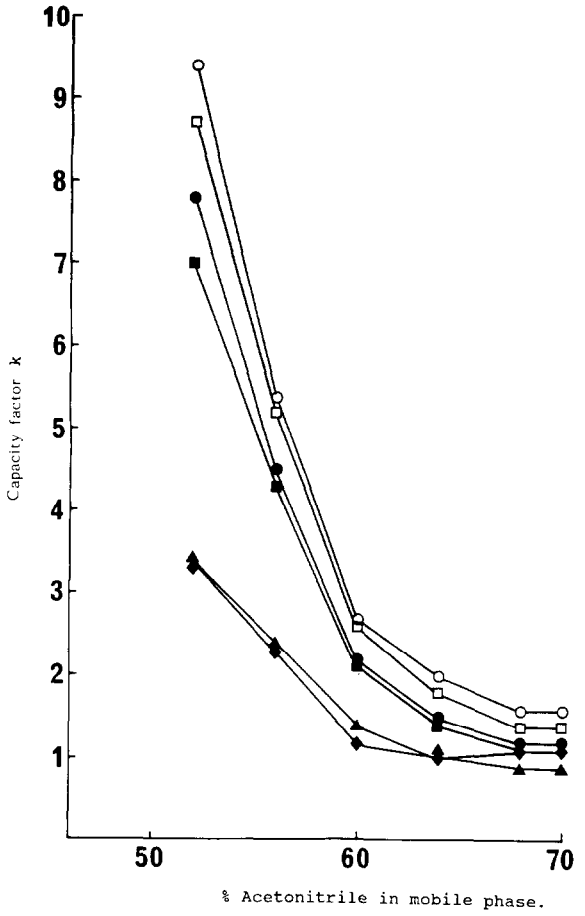

Fig. 1. Effect of acetonitrile composition on the retention of MET, PYRM and isomeric N-oxides. (○) MET; (●) MET 1N-oxide; (◆) MET 3N-oxide; (□) PYRM; (■) PYRM 1N-oxide; (▲) PYRM 3N-oxide. Mobile phase: acetonitrile–water mixtures containing 6.6 m*M* sodium dioctylsulphosuccinate pH 3; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm).

Addition of small amounts (about 4%, v/v) of tetrahydrofuran to the mobile phase has improved peak symmetry [14] of certain amines. Similarly, incorporation of secondary ions such as ammonium nitrate improves peak symmetry and reduces tailing [15,16]. The second approach was favoured since the presence of perchloric acid, a powerful oxidising agent, in the mobile phase could result in peroxide formation with tetrahydrofuran.

The effect of different concentrations of ammonium nitrate in the mobile phase on retention is shown in Fig. 2. Increasing the ammonium nitrate concentration from 1 to 20 m*M* markedly reduced retention of PYRM, MET and their 1N-oxides. This effect, commencing at 5 m*M* for the 3N-oxides, improved peak symmetry of both isomeric N-oxides.

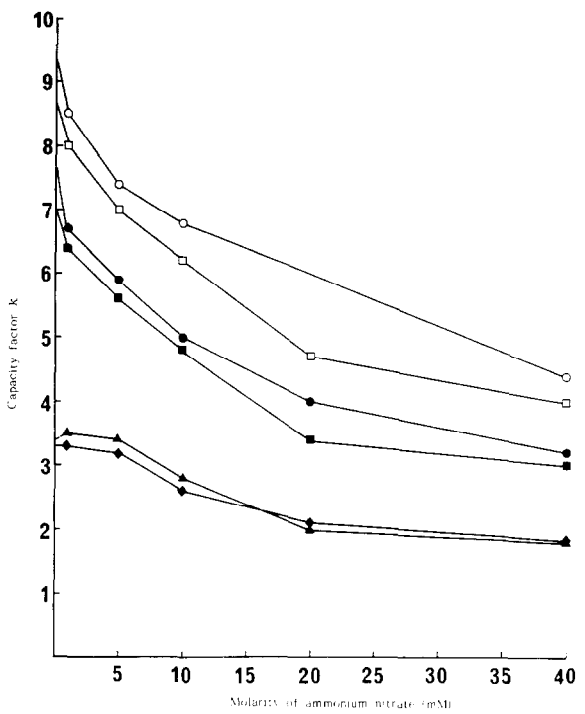

Fig. 2. Effect of ammonium nitrate concentration on the retention of MET, PYRM and isomeric N-oxides. (○) MET; (●) MET 1N-oxide; (◆) MET 3N-oxide; (□) PYRM; (■) PYRM 1N-oxide; (▲) PYRM 3N-oxide. Mobile phase: 52% acetonitrile in water containing 6.6 m*M* sodium dioctylsulphosuccinate pH 3; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm).

The effect of sodium dioctylsulphosuccinate concentration on retention is shown in Fig. 3. As expected, retention was increased with higher concentrations of dioctylsulphosuccinate. At 60% acetonitrile content, MET and its N-oxides were well resolved with 26 m*M* sodium dioctylsulphosuccinate, however, there was slight tailing of the 3N-oxide peak. The isomeric N-oxides of PYRM eluted as shoulder peaks with this mobile phase. Resolution was further improved by reducing the acetonitrile content, good resolution being obtained at 52% acetonitrile with 26 m*M* ion-pairing concentration. This mobile phase was found to achieve optimum separation of both MET and PYRM and their isomeric 1N- and

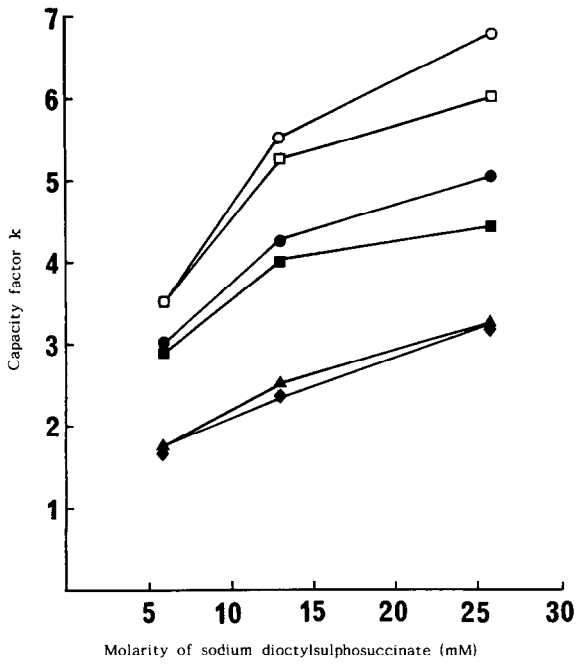

Fig. 3. Effect of sodium dioctylsulphosuccinate concentration on the retention of MET, PYRM and isomeric N-oxides. (○) MET; (●) MET 1N-oxide; (◆) MET 3N-oxide; (□) PYRM; (■) PYRM 1N-oxide; (▲) PYRM 3N-oxide. Mobile phase: 60% acetonitrile in water pH 3; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm).

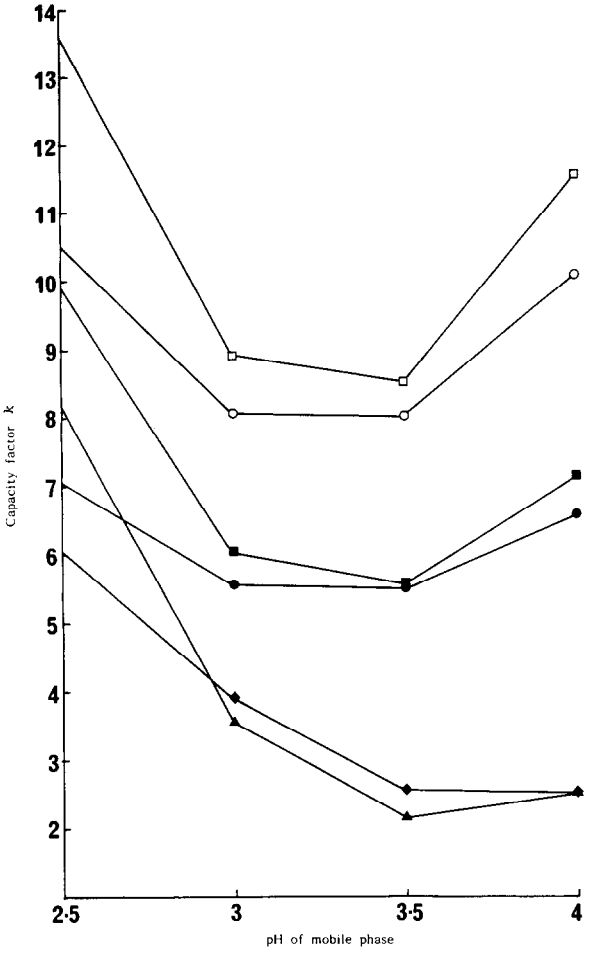

Fig. 4. Effect of mobile phase pH on the retention of MET, PYRM and isomeric N-oxides. (○) MET; (●) MET 1N-oxide; (◆) MET 3N-oxide; (□) PYRM; (■) PYRM 1N-oxide; (▲) PYRM 3N-oxide. Mobile phase: 52% acetonitrile in water containing 26 m*M* sodium dioctylsulphosuccinate and 20 m*M* ammonium nitrate, pH adjusted with perchloric acid; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm).

3N-oxides, peak symmetry being improved by the incorporation of 20 m*M* ammonium nitrate in the mobile phase.

The effect of mobile phase pH on the separation of MET, PYRM and their isomeric N-oxides using this ion-pairing system was investigated and the results are shown in Fig. 4. As expected, the results indicated a marked dependency of retention on mobile phase pH. For the parent compounds and their 1N-oxides, the pH effect was greatest in the pH region 2.5–3.0 when retention was decreased; this effect reached a plateau between pH 3.0 and pH 3.5, followed by an increased retention from pH 3.5 to pH 4.0, *i.e.* a triphasic dependency was observed. Decreased retention was observed for the 3N-oxides across the pH range 2.5–4.0. Optimum resolution with good peak symmetry occurred at pH 3.1–3.2 and chromatograms of synthetic and metabolically formed isomeric N-oxides of MET and PYRM are shown in Figs. 5 and 6.

Recovery of the N-oxides from incubation media was greater than 90% under the conditions used, calibration peak-height ratio/concentration graphs were linear in the concentration range employed and linear regression analysis of the plots gave correlation coefficients better than 0.998. The minimum detectable limits with good signal-to-noise ratio was found to be 0.5 nmol

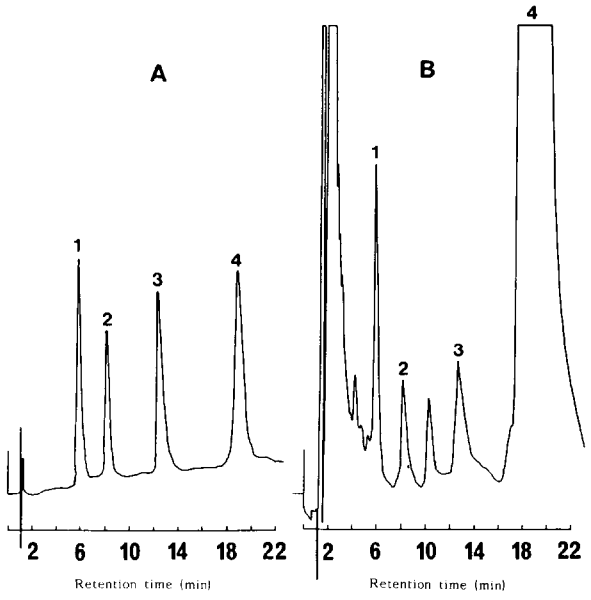

Fig. 5. HPLC of the *in vitro* metabolism of MET by hamster hepatic washed microsomes (B) and authentic MET N-oxides (A). Peaks: 1 = trimethoprim (internal standard); 2 = MET 3N-oxide; 3 = MET 1N-oxide; 4 = MET. Mobile phase: 52% acetonitrile in water containing 26 m*M* sodium dioctylsulphosuccinate and 20 m*M* ammonium nitrate pH 3.1; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm); sensitivity: 0.16 a.u.f.s.

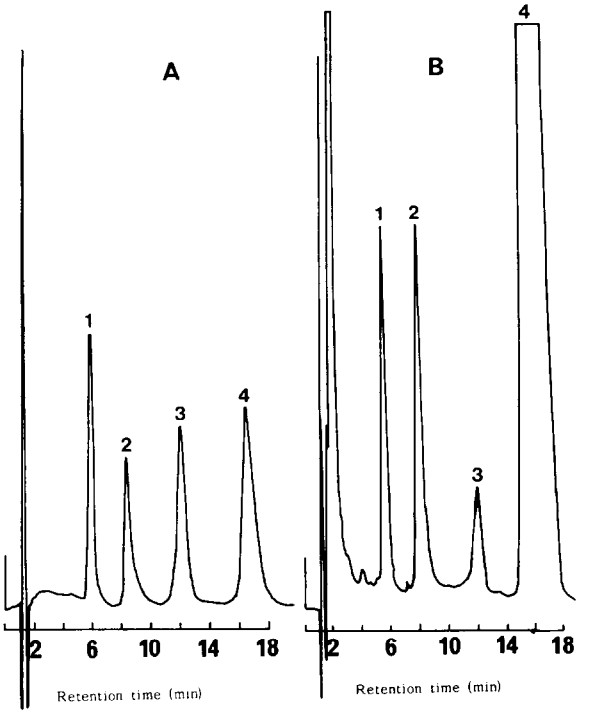

Fig. 6. HPLC of the *in vitro* metabolism of PYRM by hamster hepatic washed microsomes (B) and authentic PYRM N-oxides (A). Peaks: 1 = trimethoprim (internal standard); 2 = PYRM 3N-oxide; 3 = PYRM 1N-oxide; 4 = PYRM. Mobile phase: 52% acetonitrile in water containing 26 m*M* sodium dioctylsulphosuccinate and 20 m*M* ammonium nitrate pH 3.1; flow-rate: 1.5 ml/min; column: μBondapak C_{18} (10 μm); sensitivity: 0.16 a.u.f.s.

per incubate for both MET and PYRM isomeric 1N- and 3N-oxides. HPLC of microsomal incubates following *in vitro* metabolic studies demonstrated that MET and PYRM eluted as large broad peaks. The possibility of other compounds coeluting with MET and PYRM was recognised, but was not significant in our application since only quantitation of the N-oxide metabolites was required. Specificity of the method with regard to the N-oxides was confirmed following HPLC of control incubates which lacked either substrate (MET or PYRM), cofactors or microsomal preparation. The method has been shown to be suitably specific and sensitive for quantitation of the N-oxide metabolites of both MET and PYRM produced during *in vitro* metabolic studies.

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