Journal of Chromatography, 415 (1987) 188–196
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3501

Note

Determination of the isoniazid metabolite monoacetylhydrazine in urine by high-performance liquid chromatography

PETER J. JENNER* and GORDON A. ELLARD

National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA (U.K.)

(First received June 26th, 1986; revised manuscript received October 9th, 1986)

Isoniazid (INH, isonicotinylhydrazine) is the most widely used and potent antituberculosis drug. It is a key component of all the most effective regimens currently employed in the chemotherapy of pulmonary tuberculosis [1,2]. Furthermore, it is cheap and excellently tolerated. Serious side-effects attributed to INH treatment are rare and the most important toxic manifestation, hepatitis, occurs with an incidence of only ca. 1–2% [3,4].

The principal route of elimination of INH in humans is by acetylation to acetylisoniazid. The rate of acetylation of INH is genetically controlled and the great majority of people can be clearly characterized as being either rapid or slow acetylators of the drug. Acetylisoniazid is then hydrolysed to isonicotinic acid, which is conjugated with glycine to form isonicotinylglycine, and monoacetylhydrazine (MAH), which is then polymorphically acetylated to the terminal metabolite diacetylhydrazine [5]. Although there is evidence for the direct conversion of INH into isonicotinic acid [6], it seems unlikely that significant amounts of hydrazine are liberated in vivo since, although hydrazine is a powerful carcinogen in experimental animals [5], studies carried out among thousands of tuberculosis patients treated with INH have provided no evidence of carcinogenicity [7,8].

Studies of the acute toxicity of INH and its metabolites acetylisoniazid, MAH and diacetylhydrazine in the mouse, guinea pig, dog and monkey [9–14] have shown that acetylisoniazid and diacetylhydrazine are far less toxic than INH and MAH, suggesting that toxicity is likely to be associated with a potentially free hydrazine grouping. The only metabolite of INH in humans likely to be of potential toxicological significance would therefore appear to be MAH.

Mitchell et al. [15] have suggested that rapid acetylators are at greater risk than slow acetylators in developing INH-related liver damage, and postulated

0378-4347/87/$03.50

that this is due to microsomal activation of MAH to form potent acylating agents capable of causing liver necrosis [16]. They argued that after receiving INH, rapid acetylators would form MAH more rapidly via acetylisoniazid than slow acetylators, and suggested that as a consequence rapid acetylators would be exposed to greater amounts of the MAH and MAH-derived metabolites. The pharmacological basis for this conclusion, however, has been disputed [3,4,17–19] principally because it ignores evidence for the polymorphic acetylation of MAH in humans [6] and the prediction that as a result blood levels and tissue exposures of MAH should be similar in both acetylator phenotypes during treatment with INH [20].

Clinical and experimental investigations into the potential role of MAH in the genesis of INH-induced liver damage have been hampered by the lack of sufficiently sensitive, specific methods for estimating MAH in body fluids. Specific gas chromatographic (GC) [21] and gas chromatographic–mass spectrometric (GC–MS) [22] methods have been described for the estimation of MAH in urine, but these were generally only capable of measuring concentrations of down to ca. 0.4 μg/ml. Lauterburg et al. [23] have, however, described a much more sensitive GC–MS method for plasma MAH determination with a detection limit of ca. 0.01 μg/ml.

In view of the limitations in sensitivity of these methods or availability of GC–MS facilities, we explored the potential use of high-performance liquid chromatography (HPLC) for estimating MAH.

This paper describes the development of a simple, sensitive and selective HPLC method for the simultaneous determination of MAH and INH in urine samples involving the separation of their salicylaldehyde hydrazones by reversed-phase chromatography. The derivatisation procedure was based on a modification [6] of the procedure of Scott and Wright [24] for the fluorometric determination of INH. The chromatographic system was developed from that originally described by Abdou and Medwick [25] for measuring MAH salicylaldehyde hydrazone. In this paper we also describe the application of our HPLC method to preliminary studies of the urinary excretion of INH and MAH by a small group of healthy volunteers after the ingestion of oral doses of INH.

Since our studies were completed, Von Sassen et al. [26] published a reversed-phase HPLC procedure for the determination of MAH and INH as their *m*-chlorobenzoyl derivatives by a method involving a series of solvent extraction steps. It was claimed that concentrations of down to ca. 0.04 and 0.8 μg/ml MAH could be estimated in plasma and urine, respectively.

EXPERIMENTAL

Synthesis of reference compounds and internal standards

The synthesis, purification or sources of INH and its metabolites, acetylisoniazid, isonicotinic acid, isonicotinylglycine, diacetylhydrazine and the INH hydrazone of pyruvic acid have been described previously [27]. The α-keto-glutaric acid hydrazone of INH was a gift from Dr. R. Sarma. Since MAH and other hydrazides of simple aliphatic acids tend to be low-melting deliquescent solids,

the free bases were converted into their non-deliquescent crystalline hydrochlorides for use as analytical standards. MAH (Aldrich, Gillingham, U.K.) was first dried to dryness in a vacuum desiccator and then converted into its hydrochloride by dissolving in diethyl ether and adding an equal volume of a solution of hydrogen chloride in diethyl ether. The crystalline material that formed was filtered off and then resuspended in dry tetrahydrofuran to remove most of the remaining traces of absorbed moisture. After refiltering, the crystals of MAH hydrochloride (MAH·HCl) were dried overnight at 85 °C (melting point 140–141 °C).

Monobutyrylhydrazine (MBH) was synthesized for use as an internal standard by a method based on that originally reported by Smith [28]. Hydrazine hydrate (20 ml) was heated to 50 °C and 45.5 ml of ethyl butyrate (Aldrich) were added dropwise with stirring over 3 h followed by 20 ml of ethanol. After refluxing overnight, the solvent was removed under vacuum to give a thick oil, which on refrigeration for 24 h yielded 31 g of white crystalline MBH (yield 87%). The hydrochloride of MBH (MBH·HCl) was then prepared in the same way as used for MAH·HCl and recrystallised from methanol–diethyl ether (melting point 152–153 °C). Stock aqueous solutions (1 mg/ml) of INH, MAH·HCl and MBH·HCl were stable for at least six months when stored at 4 °C.

Preparative amounts of the salicylaldehyde hydrazones of INH, MAH and MBH were synthesized for use in developing the chromatographic system and estimating the efficiencies of the derivatisation and the extraction procedures. Gram amounts of INH, MAH·HCl or MBH·HCl were shaken with a slight excess of salicylaldehyde in warm ethanol after the procedures described by Yale et al. [29]. The hydrazones separated out rapidly and were purified by recrystallisation from ethyl acetate or ethanol–ethyl acetate (1:1).

Purification of reagents and solvents

Since commercial salicylaldehyde (Aldrich) contained impurities that interfered with the HPLC method devised for the determination of MAH and INH, it was purified by forming its bisulphite adduct, recrystallising and hydrolysing back to the aldehyde. Salicylaldehyde (50 ml) was successively washed twice with 10% sodium bicarbonate, water, 0.1 *M* hydrochloric acid, and finally water again. It was then stirred overnight in a 25% solution of sodium metabisulphate in aqueous ethanol (7:3, v/v) and the resulting crystals were filtered and liberally washed with ethanol. The bisulphite adduct was then decomposed by warming in 10% sodium bicarbonate and the liberated aldehyde extracted into diethyl ether. After drying the extract over anhydrous sodium sulphate, the ether was removed under vacuum and the salicylaldehyde was vacuum-distilled at 42 °C and stored at 4 °C in a dark glass bottle. Subsequent HPLC using the system described below gave a single peak.

HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, U.K.), and high-purity water was obtained from a Fi-stream all-glass still (Fisons, Loughborough, U.K.). All other chemicals were of analytical grade and were used as received.

Derivatisation and extraction

Estimations of MAH were carried out using undiluted urine samples, while those of INH employed dilutions of up to 100-fold such that final concentrations were within the range of the standard curve (1.0–10 μg/ml). Aliquots (1 ml) of appropriately diluted urine samples were pipetted into stoppered 10-ml test-tubes and 0.1 ml of 20 μg/ml aqueous MBH·HCl was added as internal standard. The samples were derivatised by adding 0.3 ml of an ethanolic solution of 0.1% (v/v) salicylaldehyde and 0.5 ml of 10% (v/v) aqueous acetic acid and heating for 15 min at 60°C in a water-bath. After cooling to ca. 30°C, 1.5 ml of 1 *M* K_2HPO_4 were added and the salicylaldehyde hydrazones extracted into 5 ml of diethyl ether. After centrifugation, the organic phase was transferred to a fresh centrifuge tube and shaken with 1 ml of 1 *M* K_2HPO_4 to remove most of the remaining salicylaldehyde reagent. After centrifugation the washed ether extract was transferred to a tapered test-tube and the solvent, together with any remaining traces of the salicylaldehyde reagent, was evaporated under a stream of nitrogen at 40°C. The dried residue was stored at 4°C prior to chromatography.

Liquid chromatography

A Waters Assoc. (Northwich, U.K.) chromatograph was used, consisting of Model M6000A pump, with a U6K valve injector and a Model 440 UV absorbance detector, set at 280 nm. The 250 mm×5 mm I.D. reversed-phase column containing 5-μm ODS-Hypersil (Shandon Southern, Runcorn, U.K.) was fitted with a 70 mm×2 mm I.D. guard column, dry-packed with 30–37 μm CO:Pell ODS (Whatman, Maidstone, U.K.). The mobile phase was 0.02 *M* aqueous potassium dihydrogen phosphate–acetonitrile (3:2) delivered at 1.5 ml/min. Dried urine extracts were dissolved in 100 μl of mobile phase and duplicate 25-μl aliquots were injected.

Calibration curves

Blank urine was spiked to give concentrations of 0, 0.1, 0.2, 0.5, 1, 2.5 and 5 μg/ml MAH·HCl and 0, 0.2, 0.4, 1, 2, 5 and 10 μg/ml INH. Duplicate 1-ml samples were then treated, extracted and chromatographed after the addition of 0.1 ml of 20 μg/ml MBH·HCl as internal standard as described above. The mean ratios of the peak heights of INH and MAH to that of the internal standard for the dual injections were plotted against concentrations, and the best straight line and standard errors of the slopes and intercepts calculated by the least-squares method. The equation of the calibration curve for MAH was expressed in terms of the free base.

Selectivity

The selectivity of the method was assessed by testing aqueous solutions (1 mg/ml) of all the known metabolites of INH together with the antituberculosis drugs ethambutol, *p*-aminosalicylic acid and streptomycin, as well as solutions containing 100 μg/ml rifampicin or thiacetazone in aqueous ethanol (9:1). Any compound that co-eluted with the salicylhydrazones of INH, MAH or MBH was considered to interfere.

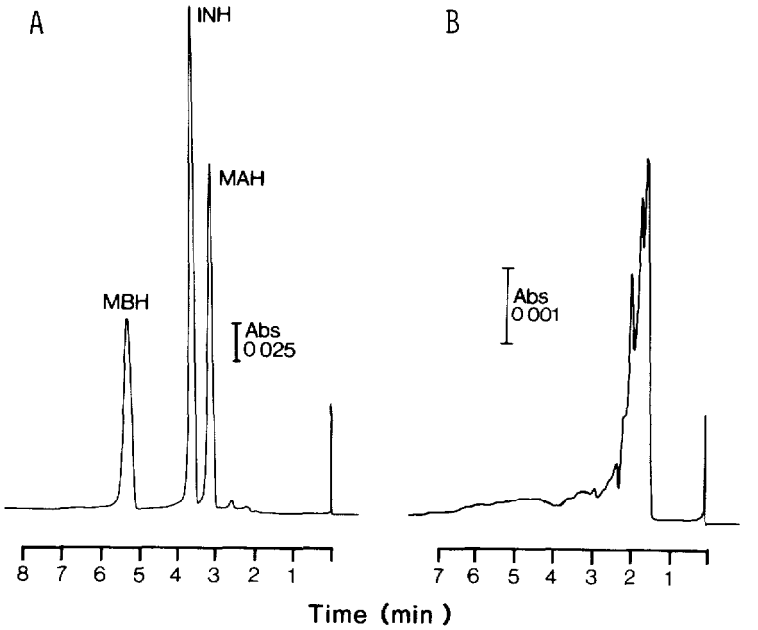

Fig. 1. (A) Chromatogram of an extract of a urine sample from the slow acetylator obtained 12–14 h after a single oral dose of 300 mg of INH. (B) Chromatogram of an extract of a pretreatment urine sample.

Urinary excretion studies

The HPLC method was applied to study the kinetics of the urinary excretion of INH and MAH after the ingestion of therapeutic doses of INH by a number of healthy volunteers. All doses of INH were swallowed on an empty stomach. In the first study a slow acetylator ingested 300 mg of INH. Hourly urine collections were made from 0–3 h, 2-h collections from 3–11 h and 23–37 h and pooled collections from 11–14 h, 14–23 h and 37–47 h, respectively. Hourly, 2-h, 3-h and 12-h urine collections were made from a rapid acetylator who swallowed 600 mg of INH, from 0–2 h, 2–6 h, 6–12 h and 12–48 h, respectively. In the second study, cumulative 0–24 h urine collections were made from seven slow and six rapid acetylators after the ingestion of single 300-mg INH doses. Aliquots of urine were stored at $-20°C$ until analysis. The acetylator phenotype of the volunteers had previously been established using sulphadimidine [17].

RESULTS

Analytical procedure

The separation of the salicylhydrazones of MAH, INH and MBH (the internal standard) are illustrated by the chromatogram of the extract of the urine collection obtained 12–14 h after ingestion of 300 mg of INH by the slow acetylator (Fig. 1A). The elution volumes of the three hydrazones were 4.8, 5.4 and 8.0 ml, respectively. All three compounds were baseline-separated and no traces of the salicyaldehyde reagent (elution volume 8.4 ml) were to be seen. Pretreatment

TABLE I

EQUATIONS OF CALIBRATION CURVES

Equation $y=mx+c$, where y is the ratio of the peak height of INH or MAH to that of the internal standard, m is the slope, x is the concentration of INH or MAH (expressed as free base), and c is the intercept.

Compound	Concentration range (μg/ml)	Slope (mean ± S.E.)	Intercept (mean ± S.E.)
MAH	0.1- 5*	1.310 ± 0.008	−0.021 ± 0.011
INH	0.2-10	0.689 ± 0.004	−0.114 ± 0.019

*Concentration of hydrochloride.

urine samples contained no interfering peaks (Fig. 1B). The equations for the calibration curves for the determination of MAH and INH are given in Table I. Replicate errors over the whole concentration range were very similar and averaged 1.5% for MAH and 2.7% for INH. The response of the detector to directly injected amounts of both salicylhydrazones was linear over a 500-fold range and as little as 5 ng of each derivative could be reliably detected. The peak heights of the directly injected salicylhydrazones were compared with those obtained after applying the extraction procedure to the salicylhydrazones and with the hydrazides when taken through the entire derivatisation and extraction procedure. It was concluded that the efficiency of salicylaldehyde hydrazone formation averaged 75 and 80% for MAH and INH, respectively, and that the extraction of the hydrazones was 78 and 68% complete, giving overall recoveries for the whole analytical procedure of ca. 59 and 54%, respectively, for the two compounds. The limit of detection of MAH was estimated to be ca. 0.01 μg/ml. Estimations of isoniazid concentrations of less than 0.1 μg/ml, however, appeared to be very unreliable on account of highly variable losses onto glassware of its salicylhydrazone.

Selectivity

No interference was encountered from the antituberculosis drugs ethambutol, *p*-aminosalicylic acid, streptomycin or thiacetazone. However, rifampicin co-eluted with the salicylhydrazone of MBH and gave a peak of ca. 10% of that of the internal standard. Rifampicin could, however, be completely removed prior to determining MAH and INH by extracting urine samples with three volumes of amyl alcohol [30]. As was anticipated, the isoniazid metabolites, acetylisoniazid, isonicotinic acid, isonicotinylglycine and diacetylhydrazine did not interfere with the method since they do not possess a free hydrazine group and are incapable of reacting with salicylaldehyde. Hydrazine did not give rise to a peak, presumably because salicylaldehyde azine was retained on the column. However, the pyruvic and α-ketoglutaric hydrazone metabolites of isoniazid gave identical results to isoniazid (on a molar basis), indicating that the method measures acid-labile INH like other methods for measuring the drug based on hydrazone formation [21,27]. It would therefore seem highly likely that similar acid-labile metabolites of MAH [5] would also be measured as MAH [21].

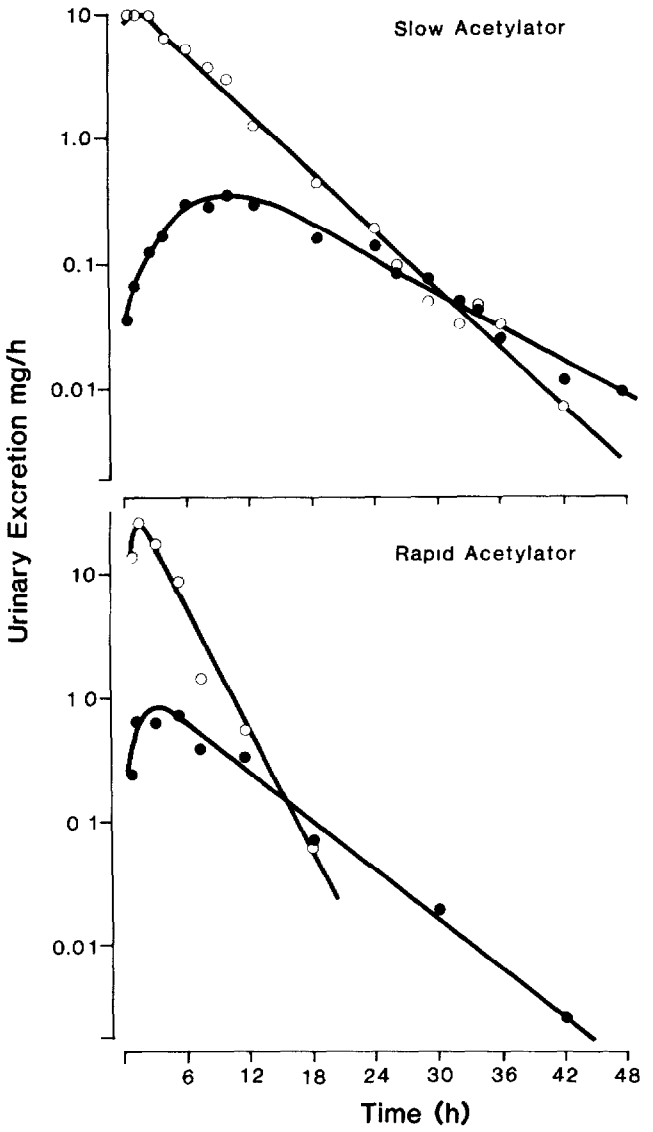

Fig. 2. Urinary excretion of acid-labile INH (○) and MAH (●) in a slow acetylator following a single oral dose of 300 mg of INH and in a rapid acetylator after a single oral dose of 600 mg of INH.

Urinary excretion of acid-labile MAH and INH

The kinetics of the urinary excretion of acid-labile INH and MAH in a slow acetylator following ingestion of 300 mg of INH are illustrated in Fig. 2. The maximum rate of excretion of acid-labile INH in the slow acetylator occurred within the first hour and declined thereafter at a rate equivalent to a half-life of 3.8 ± 0.1 h. By contrast the rate of urinary excretion of acid-labile MAH increased slowly, reaching a maximum at ca. 10 h and then declined over the period 12.5–42 h at a rate equivalent to an apparent half-life of 6.5 ± 0.5 h. In the rapid acetylator, after 600 mg of INH, excretion rates of acid-labile INH declined at a rate equiv-

alent to a half-life of 1.8 ± 0.2 h, while maximal rates of excretion of acid-labile MAH were encountered at 6 h and declined from 7.5 to 42 h with an apparent half-life of 4.6 ± 0.3 h.

Over the 48-h period the slow acetylator excreted 26% of the isoniazid dose as acid-labile INH and 3.7% as acid-labile MAH (2.5% within the first 24 h), while the proportions excreted by the rapid acetylator were 16 and 2.6% (2.5% within 24 h), respectively. The cumulative 0–24 h urinary excretion of acid-labile MAH in the six rapid acetylators averaged 1.3% (S.E. 0.6%) of the dose, which was significantly less than that of the seven slow acetylators ($2.9 \pm 0.6\%$) ($P = 0.005$, Mann–Whitney non-parametric test). These results are similar to previously published studies [31,32] using a GC method [21] for measuring the excretion of acid-labile MAH by rapid and slow acetylators. The 0–24 h urinary excretion of acid-labile INH in the slow acetylators was about four times that of the rapid acetylators, a difference that, as anticipated, was highly significant ($P = 0.003$).

DISCUSSION

The HPLC method described for the determination of urinary concentrations of acid-labile MAH and INH is sensitive and relatively simple. None of the other metabolites of INH interfere with the method and its specificity as regards other antituberculosis drugs is good. Although the method measures MAH and INH together with their acid-labile hydrazone metabolites, this is almost certainly advantageous since it is thought that the hydrazones are essentially artifacts formed chemically in the bladder and not enzymatically catalysed by the liver.

The method's limit of detection (to ca. 0.01 μg/ml MAH) is at least an order of magnitude lower than that of alternative GC or HPLC procedures [21,26] and similar to that of the most sensitive GC–MS method [23]. However, in addition to requiring far less elaborate and expensive equipment than GC–MS, it is also much less time-consuming, the average assay time being only ca. 15 min per sample. It can also be applied to the concomitant measurement of acid-labile INH in the samples.

The method has been successfully applied to study the kinetics of the urinary excretion of acid-labile MAH and INH after the ingestion of INH by a slow and rapid acetylator and to studies on their cumulative excretion by a panel of the two phenotypes.

The results obtained are broadly in accord with those obtained in plasma level and urinary excretion studies by both GC and GC–MS methods [31–33]. More extensive studies of the kinetics of the urinary excretion of acid-labile MAH undertaken to examine its postulated role in INH-induced hepatotoxicity will be reported elsewhere.

REFERENCES

1 W. Fox, Bull. Int. Union Tuberc., 60 (1985) 40.
2 W. Fox, Br. J. Dis. Chest, 75 (1981) 331.
3 D.J. Girling, Drugs, 23 (1982) 56.

4 D.J. Girling, Tubercle, 59 (1978) 13.
5 H. Druckrey, Xenobiotica, 3 (1973) 271.
6 G.A. Ellard and P.T. Gammon, J. Pharmacokinet. Biopharm., 4 (1976) 83.
7 H. Stott, J. Peto, R. Stephens, W. Fox, I. Sutherland, A.F. Foster-Carter, H.D. Teare and J. Fenning, Tubercle, 57 (1976) 1.
8 J.L. Glassroth, M.C. White and D.E. Snider, Am. Rev. Resp. Dis., 116 (1977) 1065.
9 J. Bernstein, W.A. Lott, B.A. Steinberg and H.L. Yale, Am. Rev. Tuberc., 65 (1952) 357.
10 H.B. Hughes, J. Pharmacol. Exp. Ther., 109 (1953) 444.
11 E.H. Jenney and C.C. Pfeiffer, J. Pharmacol. Exp. Ther., 122 (1958) 110.
12 M.O. Tirunarayanan and W.A. Vischer, Experientia, 14 (1958) 22.
13 H. McKennis, A.S. Yard, J.S. Weatherby and J.A. Hagy, J. Pharmacol. Exp. Ther., 126 (1959) 106.
14 A.S. Yard and H. McKennis, J. Med. Pharm. Chem., 5 (1962) 196.
15 J.R. Mitchell, U.P. Thorgeirsson, M. Black, J.A. Timbrell, W.R. Snodgrass, W.Z. Potter, D.J. Jallow and H.R. Keiser, Clin. Pharmacol. Ther., 18 (1975) 70.
16 J.R. Mitchell, H.J. Zimmerman, K.G. Ishak, U.P. Thorgeirsson, J.A. Timbrell, W.R. Snodgrass and S.D. Nelson, Ann. Int. Med., 84 (1976) 181.
17 G.A. Ellard and P.T. Gammon, Br. J. Clin. Pharmacol., 4 (1977) 5.
18 W.W. Weber and D.W. Hein, Clin. Pharmacokinet., 4 (1979) 401.
19 W.W. Weber, D.W. Hein, A. Litwin and G.M. Lower, Fed. Proc., Fed. Am. Soc. Exp. Biol., 42 (1983) 3086.
20 G.A. Ellard, Bull. Int. Union Tuberc., 51 (1976) 143.
21 J.A. Timbrell, J.M. Wright and C.M. Smith, J. Chromatogr., 138 (1977) 165.
22 A. Noda, K.-Y. Hsu, Y. Aso, K. Matsumyama, S. Iguchi and M. Hirata, J. Chromatogr., 230 (1982) 345.
23 B.H. Lauterburg, C.V. Smith and J.R. Mitchell, J. Chromatogr., 224 (1981) 431.
24 E.M. Scott and R.C. Wright, J. Lab. Clin. Med., 70 (1967) 355.
25 H.M. Abdou and T. Medwick, J. Org. Chem., 43 (1978) 15.
26 W. von Sassen, M. Castro-Parra, E. Musch and M. Eichelbaum, J. Chromatogr., 338 (1985) 113.
27 G.A. Ellard, P.T. Gammon and S.M. Wallace, Biochem. J., 126 (1972) 449.
28 P.A.S. Smith, Org. Reactions, 3 (1946) 337.
29 H.L. Yale, K. Losee, J. Martins, M. Holsing, F.M. Perry and J. Bernstein, J. Am. Chem. Soc., 75 (1953) 1933.
30 S. Sunahara and H. Nakagawa, Chest, 61 (1972) 526.
31 J.A. Timbrell, J.M. Wright and T.A. Baillie, Clin. Pharmacol. Ther., 22 (1977) 602.
32 J.A. Timbrell, B.K. Park and S.J. Harland, Human Toxicol., 4 (1985) 279.
33 D.H. Lauterburg, C.V. Smith, E.L. Todd and J.R. Mitchell, J. Pharmacol. Exp. Ther., 235 (1985) 566.