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Short Communication

Improved high-performance liquid chromatographic method for the determination of ethylmorphine and its metabolites in microsomal incubations and cell culture media

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ABSTRACT

Ethylmorphine N-demethylation is used as a marker pathway in studies of rat cytochrome P450 3A and 2C11 biotransformations. At present, microsomal activities are generally measured by a colorimetric determination of the formed formaldehyde. In the present study, a high-performance liquid chromatographic method of separating and quantifying both the N-demethylated (norethylmorphine) and the O-de-ethylated (morphine) metabolites is described. Either samples are extracted with ethyl acetate or proteins are precipitated with zinc sulphate–barium hydroxide. Separation is achieved on a CN reversed-phase column, using a mobile phase of phosphate buffer (pH 4.5)–acetonitrile (90:10, v/v). At a flow-rate of 1.5 ml/min, the analysis time is 30 min. The limit of detection (ultraviolet, 210 nm) for ethylmorphine and its metabolites is 0.5 μg/ml.

INTRODUCTION

Ethylmorphine (EtM), formerly used as a narcotic analgesic, is now a test substrate in pharmacology and toxicology. It is generally used in bio-

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transformation studies *in vitro* because of the cytochrome P450 enzyme-specific product formation. Fig. 1 shows EtM metabolism through N-demethylation and O-de-ethylation, yielding norethylmorphine (NEtM) and morphine (Mo), respectively [1,2]. In rats, N-demethylation is mediated through enzymes from the P450 3A and 2C subfamilies and O-de-ethylation through P450 2D subfamily enzymes [3]. In addition to the oxidative pathways, O-glucuronidation of the parent compound and its dealkylated metabolites may occur, as is the case for the EtM analogue codeine [4].

In the vast majority of reports dedicated to EtM metabolism, only N-demethylation was studied. This biotransformation pathway is of interest because it is considered to be a marker for P450 3A and 2C11 activity [2], it is sexually differentiated in rats and mice [5–7], and it can be determined by a relatively simple spectrophotometric method, published by Nash [8]. In this method, formaldehyde formed in microsomal incubations reacts with acetylacetone, yielding a yellow product (maximum absorption at 412 nm). De-ethylation does not yield formaldehyde and, therefore, cannot be determined by the Nash method. The colorimetric method has limited sensitivity [2].

Determination of both EtM metabolites adds to the value of the assay since the activities of several P450 enzymes can be studied simultaneously. Moreover, direct determination of N-demethylated ethylmorphine is more specific than that of formaldehyde. Formaldehyde losses caused by further oxidation may be important. Semicarbazide is generally used as trapping agent for formaldehyde to prevent such losses. However, the use of semicarbazide should be avoided because of its inhibitory effect on biotransformation [9].

Using [1′-^{14}C]EtM, Nerland and Mannering [1] measured the formation of radiolabelled acetaldehyde to determine O-de-ethylation, in addition to N-demethylation using the Nash method. Duquette and Holtzman [2] separated and quantified labelled NEtM and Mo by thin-layer chromatography (TLC), followed by scintillation counting. Jarvi *et al.* [10] were the first to describe a reversed-phase high-performance liquid chromatographic (HPLC) method for analysis of EtM and its metabolites. A good correlation was found between results obtained with the Nash method and the ion-pair HPLC method described. The main drawback of this method is the incomplete separation of EtM and NEtM. A better separation was achieved by Ladona *et al.* [11]. These authors used a CN-coated reversed-phase column without ion pairing. The presence of background peaks in their chromatograms clearly shows the necessity for a full separation of EtM and NEtM. Unfortunately, with this method Mo could not be determined.

The aim of the present study was to develop an easy routine analysis for EtM, NEtM and Mo,

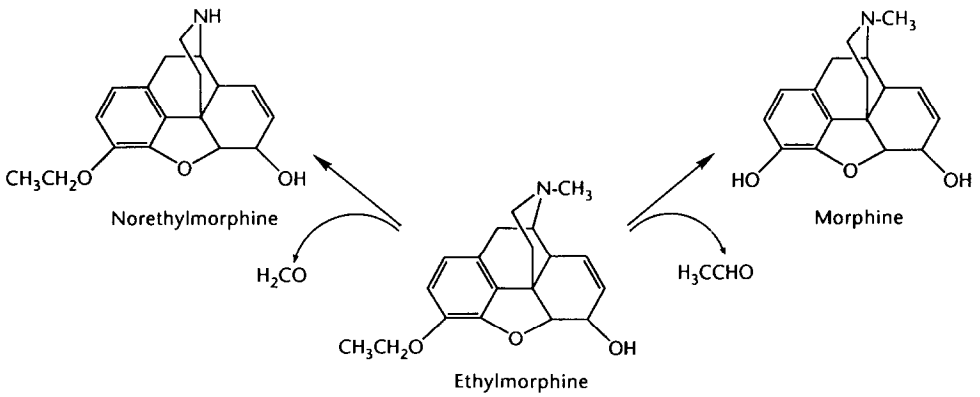

Fig. 1. N-Demethylation and O-de-ethylation of ethylmorphine, yielding norethylmorphine and morphine, respectively. N-Demethylation results from formaldehyde elimination.

interchangeable with the Nash method [8] for EtM N-demethylation. Sample preparation by liquid extraction is compared with protein precipitation without extraction. The method is applied to EtM metabolism studies in microsomal incubations and isolated hepatocytes prepared from goat liver.

EXPERIMENTAL

Reagents, solvents and materials

Hydrochloride salts of ethylmorphine and morphine were obtained from OPG (Utrecht, Netherlands) and nalorphine from Bufa (Castricum, Netherlands). Norethylmorphine was kindly donated by Dr. B. Lindström and Professor A. Rane (National Board of Health and Welfare, Uppsala, Sweden).

NADP, glucose-6-phosphate dehydrogenase (grade I, from yeast) and glucose 6-phosphate were obtained from Boehringer (Mannheim, Germany). Semicarbazide, magnesium chloride hexahydrate, zinc sulphate, barium hydroxide, Williams' Medium E and limpet acetone powder (LAP) were purchased from Sigma (St. Louis, MO, USA).

HPLC-grade acetonitrile was from Rathburn (Walkerburn, UK). Demineralized water was filtered through a Milli-Q device from Millipore (Etten-Leur, Netherlands). Potassium dihydrogenphosphate, phosphoric acid and ethyl acetate (p.a. grade) were obtained from Baker (Deventer, Netherlands).

All other chemicals were of the best available grade.

Apparatus

Spectrophotometric determinations were performed with a programmable PU 8800 UV–VIS spectrophotometer (Pye Unicam, Eindhoven, Netherlands).

The chromatographic system consisted of a Promis Autosampler from Spark Holland (Emmen, Netherlands), a 400S solvent delivery system and a 783A programmable absorbance detector set at 210 nm from Applied Biosystems (Maarssen, Netherlands), and an SP4290 integrator from Spectra Physics (San Jose, CA, USA). A 100 mm × 8 mm I.D. Resolve CN 10-μm Radial PAK cartridge column equipped with a 4 mm × 6 mm I.D. Resolve CN 10-μm guard column from Waters (Etten-Leur, Netherlands) was used. The mobile phase consisted of potassium phosphate buffer (0.05 *M*, pH 4.5)–acetonitrile (90:10 v/v). The phosphate buffer was prepared by dissolving 6.8 g of potassium dihydrogenphosphate per litre of water and adjusting the pH with phosphoric acid. It was filtered (0.45-μm filter, Millipore) and degassed under vacuum before use. The flow-rate amounted 1.5 ml/min at a backpressure of 40 bar. Analyses were performed at ambient temperature.

Animals and in vitro incubations

Clinically healthy dwarf goats, aged eight to ten months (10–14 kg), were used throughout this study. The Animal Ethics Committee of the Veterinary Faculty approved the use of the animals to study drug metabolism and its regulation in agricultural species, which includes the present study.

Microsomes were prepared by differential ultracentrifugation of homogenized liver [12]. Microsomes were incubated with 5.0 m*M* EtM for 10 min at 37°C in 2.5 ml of a 0.125 *M* phosphate buffer (pH 7.4) containing 1.0 mg of NADP, 1.5 mg of glucose 6-phosphate, 1.0 μl of glucose-6-phosphate dehydrogenase, 0.6 mg of magnesium chloride hexahydrate and, for Nash determinations, 1 mg of semicarbazide.

Goat hepatocyte isolation, culture and incubation were performed as described elsewhere [13]. Hepatocytes were incubated with 2.5 m*M* EtM in serum-free Williams' Medium E for 2 h.

The cytochrome P450 and protein contents of microsomes and hepatocyte cultures were measured according to Rutten *et al.* [12].

Sample preparation

Microsomal incubation mixtures and hepatocyte culture medium samples were processed by either liquid extraction or deproteinization.

Ethyl acetate extraction. Microsomal incubation samples (2.5 ml) were mixed with 100 μl of a

1 m*M* solution of nalorphine (internal standard) and 1.0 ml of a solution of 6.2% (w/v) boric acid and 7.5% (w/v) potassium chloride, adjusted to pH 9.0 with 4 *M* sodium hydroxide. After saturation with approximately 1 g of ammonium sulphate, samples were extracted twice with 4.0 ml of ethyl acetate. The combined organic fractions were evaporated to dryness under nitrogen. Dry residues were redissolved in 1 ml of the mobile phase. A 20-μl aliquot was injected for HPLC analysis.

Protein precipitation. Microsomal incubations (2.5 ml) were deproteinized by adding 0.5 ml of 40% (w/v) zinc sulphate and 1 ml of saturated barium hydroxide solution, mixing and centrifuging (10 min, 500 *g*), as described by Noordhoek *et al.* [7]. The supernatant was used either for formaldehyde determination according to Nash [8] or for EtM metabolite determination by HPLC.

For colorimetry, 2 ml of the supernatant were mixed with 1 ml of double-strength Nash reagent and incubated for 30 min at 60°C. Formaldehyde concentration was estimated from the 415- and 500-nm absorbance difference.

For HPLC analysis, 20 μl from the remaining part of the supernatant were directly injected in the chromatographic system.

Hepatocyte sample pretreatment. Medium samples (2.5 ml) from hepatocyte cultures were mixed with 1 ml of 0.5 *M* acetate buffer (pH 4.5) and approximately 20 mg of limpet acetone powder (a crude extract with glucuronidase and sulphatase activity). Samples were incubated for 24 h in a water bath held at 37°C. To each sample, 50 μl of 4 *M* sodium hydroxide were added. Further handling was identical to microsomal incubation samples.

RESULTS AND DISCUSSION

Chromatography

In a series of experiments, cultured goat hepatocytes were incubated with EtM. Ethyl acetate-extracted medium samples yielded the chromatograms shown in Fig. 2. Prior to extraction, glucu-

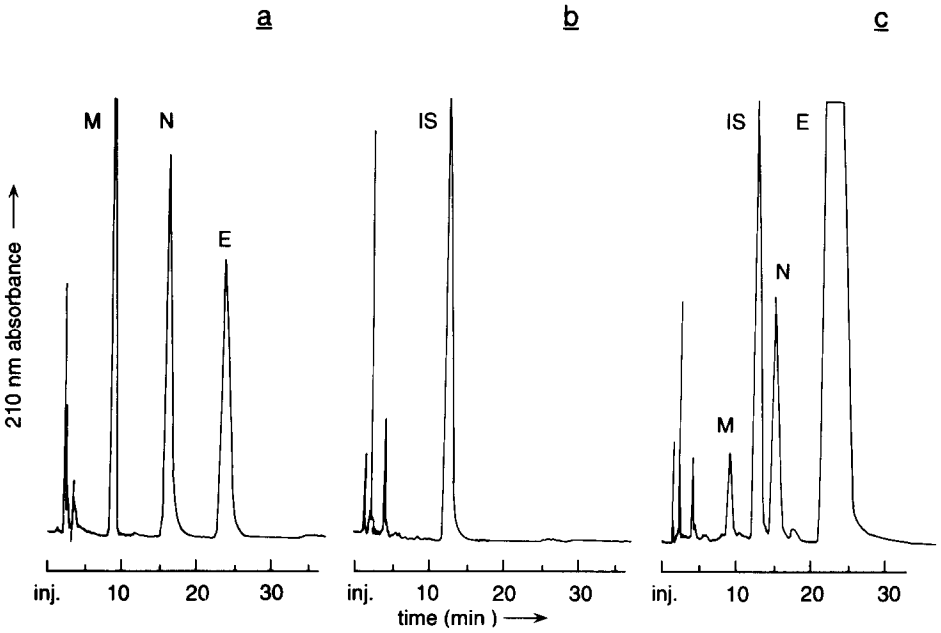

Fig. 2. Representative chromatograms (a.u.f.s. = 0.16) from (a) analysis of a reference mixture (50 μM), (b) blank hepatocyte culture medium and (c) culture medium after 2.5 m*M* ethylmorphine incubation. Samples were extracted with ethyl acetate. Elution: acetonitrile–buffer pH 4.5 (10:90, v/v); 1.5 ml/min. Peaks: N = norethylmorphine; M = morphine; E = ethylmorphine; IS = internal standard.

ronides and sulphates of the parent compound and its metabolites were deconjugated using LAP. Within 24 h incubation at 37°C, deconjugation was considered complete, since no further increase of extractable compounds could be observed. An advantageous feature of LAP is that it does not yield additional matrix peaks.

Similar analytical results (not shown) were obtained if samples from microsomal incubation mixtures were extracted with ethyl acetate. Microsomal incubations, deproteinized with zinc sulphate–barium hydroxide, yielded the chromatograms in Fig. 3. The total run time was approximately 30 min in the method as described. The analysis time could easily be reduced by slight increases in modifier concentration and flow-rate. Examples of the latter modification yielding accelerated analysis are shown in Fig. 3b, c and d.

Either method yields complete separation of EtM, its metabolites and the internal standard. Straight baselines and minor matrix disturbances are observed, even with the detection wavelength of 210 nm, adapted from Ladona *et al.* [11], which is non-selective but provides high sensitivity. Fig. 3b, c and d show an unknown matrix peak at a retention time (t_R) of 10 min that is not completely separated from NEtM, although no problems are met with quantification. The peak was often but not always (Fig. 3a) observed with microsomal incubation mixtures.

The retention of EtM and its metabolites was found to be pH-dependent. With the present method, using the same column as Ladona *et al.* [11], all peaks were rapidly eluted with 10% (v/v) acetonitrile in a 50 m*M* phosphate buffer (pH 4.5). Ladona *et al.* [11] used an eluent at pH 6.9 and, therefore, needed 45% acetonitrile for elution. Table I shows the pH-dependent increase in modifier (acetonitrile) concentration required to maintain similar retention times for EtM, NEtM and Mo. Separation of EtM and NEtM was optimal at pH > 5, whereas NEtM and Mo were better separated at pH < 6 (Table I). In the present method, pH 4.5 was chosen since nalorphine, the internal standard used, eluted between NEtM and Mo.

As with most bases, the peak shape of EtM,

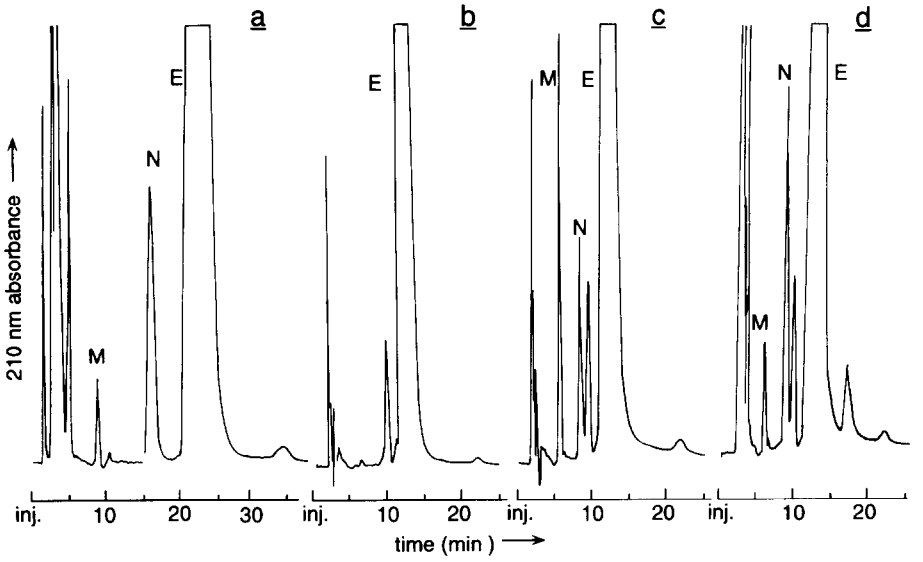

Fig. 3. Sample analysis in two different series of experiments (a *versus* b, c and d) from microsomal incubation mixtures after precipitation of proteins. (a) Microsomal ethylmorphine (E) incubation after 30 min; elution with 10% (v/v) acetonitrile (1.5 ml/min). (b) Mixture incubated for 30 min without microsomes. (c) Mixture incubated without microsomes and spiked (4 μg/ml) with norethylmorphine (N) and morphine (M). (d) Microsomal ethylmorphine incubation after 30 min. Samples in b, c and d were eluted with 11% (v/v) acetonitrile (2.0 ml/min). The nature of the peak (t_R = 10 min) found in the second series of experiments (b, c and d) is unknown.

TABLE I

MOBILE PHASE COMPOSITIONS WITH VARYING ELUENT BUFFER (pH) AND ACETONITRILE CONCENTRATION (%, v/v) YIELDING APPROXIMATELY SIMILAR RETENTION PROFILES

Flow-rate of 1.5 ml/min.

pH	Acetonitrile (%)	Retention time (min)		
		EtM	NEtM	Mo
4.5	10	16.6	14.3	8.3
5.0	15	14.0	12.0	6.9
6.0	20	16.2	11.7	8.9
6.9	30	19.6	12.1	10.4

NEtM and Mo could be improved by adding the capping agent triethylamine (TEA) to the mobile phase (0.2%, v/v). However, TEA decreases retention of the compounds of interest by a factor of 2 (results not shown).

Ethyl acetate extraction

Recovery from ethyl acetate extraction was determined using cell culture medium or incubation mixture for microsomes spiked with EtM, its metabolites and nalorphine in concentrations ranging from 5 to 500 μM (approximately 1.5–150 μg/ml). Recoveries were (eight different concentrations, in duplicate, mean ± S.D.): EtM, 79.1 ± 2.4%; NEtM, 77.4 ± 6.2%; Mo, 74.3 ± 5.9%; and nalorphine, 82.0 ± 4.2%. The recovery of NEtM and Mo (pK_a 8.0 and 9.9, respectively [14]) is dependent on the pH of the aqueous phase during liquid extraction.

Nalorphine, a structural analogue of morphine, was chosen as internal standard. The compound has an N-allyl group instead of an N-methyl group. Extraction recovery is not significantly different from that of ethylmorphine and its metabolites. Nalorphine elutes between morphine and norethylmorphine (Fig. 2). In our studies, no interfering peaks occurred in this area.

Sample pretreatment by deproteinization

Fig. 3 shows the results of the analysis of samples from microsomal incubation mixtures. Precipitating proteins and centrifugation, identical to sample pretreatment for determination of EtM N-demethylation using the Nash method, does not yield matrix peaks interfering with the peaks of interest.

No internal standard was used since co-precipitation and other sources of metabolite losses were negligible (recovery > 97%).

Linearity and limit of detection

For EtM, NEtM and Mo, calibration graphs were constructed. Linear correlations ($r \geq 0.99$) were found for concentrations from 5 to 500 μM.

In both described analytical systems, the limit of detection was 0.5 μg/ml for Mo and 1.0 μg/ml for NEtM (and EtM). If the extraction method was used, this limit could be decreased by reconstitution of the residue in a smaller volume of eluent. Concentrations of 2.0 μg/ml for Mo and 3.5 μg/ml for NEtM and EtM were considered the limit of quantitation, having an accuracy of 4% (based on four consecutive analyses).

Whereas intra-assay reproducibility was better than 3% (based on four consecutive assays), day-to-day variation ranged from 3% (protein precipitation) to 5% (ethyl acetate extraction, internal standard corrected).

HPLC analysis versus Nash method

To compare quantification of NEtM by the HPLC method described with quantification of formaldehyde using Nash's colorimetric method [8], microsomes were incubated with EtM in the presence of semicarbazide. An aliquot of pretreated sample was injected into the chromatographic system (Fig. 3). The remaining part was used directly for colorimetry. The results are shown in Fig. 4.

Microsomes from 25 male, female or castrated goats, treated or not treated with model cytochrome P450 inducers, in triplicate, were tested to achieve a broad range of metabolic activity towards EtM.

A good correlation ($r > 0.98$) between formaldehyde production and NEtM formation was found. Therefore, for EtM N-demethylation, we consider the Nash method and the present HPLC

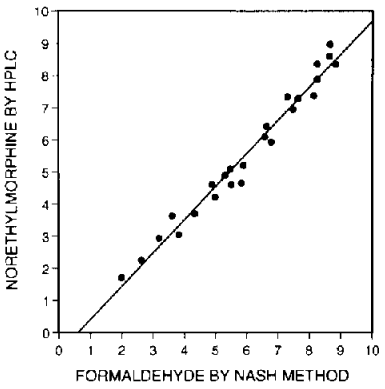

Fig. 4. Metabolite formation in goat microsomal incubations of ethylmorphine, expressed in nmol/mg protein min. Each point (mean of triplicate) represents one goat ($n = 25$). A linear correlation ($r = 0.986$, least-squares regression analysis) between formaldehyde quantification by the Nash method and norethylmorphine determination by HPLC was found.

method interchangeable. This is an important feature, necessary to relate results from metabolism studies obtained with the HPLC method presented in this study to data from the literature, which are all based on the Nash determination. Since the HPLC method provides a simultaneous determination of other metabolites, and it determines the actual metabolites rather than the by-products, it is the method of choice.

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