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Gas chromatographic–mass spectrometric analysis of alfentanil metabolites
Application to human liver microsomal alfentanil biotransformation

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Abstract

The short-acting synthetic opioid alfentanil undergoes extensive biotransformation to several metabolites. A gas chromatographic–mass spectrometric assay, using selected-ion monitoring and deuterated internal standards, was developed for quantitating the predominant metabolites of alfentanil. Optimal extraction and derivatization conditions are described. The assay was applied to the analysis of metabolites formed during alfentanil metabolism in vitro by human liver microsomes. Formation of known alfentanil metabolites was confirmed, and formation of a metabolite, not previously detected in vitro, is described. The assay represents a significant improvement over existing methods of alfentanil metabolite analysis, which use HPLC and radiochemical detection.

1. Introduction

Alfentanil (N-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(methoxymethyl)-4-piperidinyl]-N-phenyl propanamide) (ALF, Fig. 1) is a short acting, synthetic opioid that is used widely in clinical anesthesia. There is extreme and unexplained interindividual variability in ALF pharmacokinetics, exemplified by at least a 10-fold variability in plasma clearance [1–3]. Since ALF undergoes extensive hepatic metabolism, differences in ALF metabolism may underlie the variability in ALF elimination clearance [4,5]. Therefore there has been considerable interest in both in vivo and in vitro human ALF biotransformation.

ALF undergoes extensive hepatic cytochrome P450-catalyzed metabolism in humans, with less than 1% of the dose excreted unchanged [6]. ALF metabolism occurs by two predominant and possibly interdependent pathways (Fig. 1) [6,7]. The first major pathway (a) involves N-dealkylation at the piperidine nitrogen to form noralfentanil (N-[4-(methoxymethyl)-4-piperidinyl]-N-

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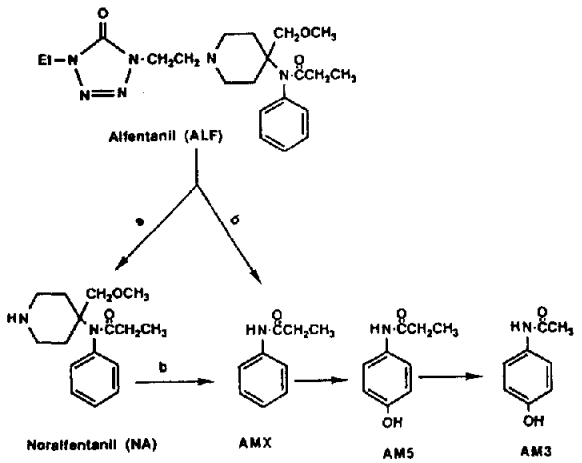

Fig. 1. Major pathways of alfentanil metabolism: (a) piperidine N-dealkylation and (b) amide N-dealkylation. The predominant in vivo metabolites are NA, AM5, and AM3 [6]. The major in vitro metabolites are NA and AMX [7].

phenylpropanamide), which is the major metabolite formed both in vivo and in vitro [6,7]. In clinical studies, noralfentanil (NA) accounted for approximately one-third of the ALF dose [6]. A small amount of N-[4-(hydroxymethyl)-4-piperidinyl]-N-phenylpropanamide (desmethylnoralfentanil, AM6), a proposed secondary metabolite of noralfentanil, was also detected in vivo [6]. The second major pathway (b) of alfentanil metabolism involves the formation of N-phenylpropanamide (AMX) and its secondary metabolites, N-(4-hydroxyphenyl)propanamide (AM5) and N-(4-hydroxyphenyl) acetamide (AM3) [6,7]. Clinically, AM5, AM3 and their glucuronide conjugates together accounted for approximately one-fourth of the ALF dose [6]. AMX was not detected in urine in vivo [6], although in vitro studies have demonstrated a rapid rate of AMX production [7]. AMX may theoretically arise from metabolism of alfentanil and/or noralfentanil.

Currently, there is no satisfactory assay for detection and quantitation of ALF metabolites. One previous investigation of ALF metabolism in vivo, measuring ALF metabolite formation, utilized ^{3}H-alfentanil and an HPLC assay for detection of radioactive metabolites [6]. This method is limited, however, by the requirement for radiolabelled ALF and because detection is restricted to those metabolites retaining the tritium label. Furthermore, administration of radiolabelled drug is not desirable for routine human clinical studies. Investigations of ALF metabolism in vitro have successfully used ^{3}H-ALF with radio-HPLC techniques [5,7]. Investigations using this assay reported only the formation of NA and AMX in vitro, but none of the other in vivo ALF metabolites [7]. In vitro investigations of ALF metabolism have also been performed using unlabelled ALF, and monitoring of substrate disappearance by gas chromatography coupled with nitrogen detection or mass spectrometry [4,8]. However this disappearance assay does not permit detection or quantitation of ALF metabolites.

The purpose of this investigation, therefore, was to develop an assay for the major ALF metabolites that does not require radiolabelled substrate. We describe a gas chromatography–mass spectrometry assay and its application to an in vitro model of human liver microsomal ALF metabolism.

2. Experimental

2.1. Chemicals

Pentafluoropropionic anhydride (PFAA), N-methyl-bistrifluoroacetamide (MBTFA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and BSTFA with 1% trimethylchlorosilane (BSTFA–TMCS) were purchased from Pierce (Rockford, IL, USA). Deuterated aniline (ring d_5) was from Aldrich (Milwaukee, WI, USA). Ethyl acetate (HPLC grade from Baker) was used for extractions and ethyl acetate (optima grade from Fisher Scientific, dried over molecular sieves) was used for sample derivatization and reconstitution.

Noralfentanil was synthesized according to previously published methods [9]. The deuterated analog of noralfentanil, labelled on the aromatic ring, was synthesized from d_5-aniline by modification of the synthetic scheme for

noralfentanil. Intermediate compounds in the synthesis were identified by thin layer chromatography (normal- and reversed-phase silica) and characterized by ^{1}H NMR (Varian XLR 300), infrared spectroscopy (Perkin-Elmer Model 1600 FTIR) and gas chromatography–mass spectrometry (GC–MS). The final compounds were purified by reversed-phase HPLC and were found to be 95% pure. The HPLC system consisted of an SSI (Scientific Systems Inc., State College, PA, USA) Model 220B pump, SSI Model 500 variable wavelength detector operated at 255 nm, Rheodyne 7125 injection port, and an Econosil (10 × 250 mm, 10 μm) reversed-phase C_{18} column (Alltech Associates, Deerfield, IL, USA). Elution was performed with an isocratic mobile phase (0.25 *M* ammonium acetate–acetonitrile (50:50) adjusted to a final pH of 8.0) at a flow-rate of 2.5 ml/min. Identification was confirmed by comparison to authentic unlabelled noralfentanil, provided by Janssen Research Foundation (Beerse, Belgium). Unlabelled and deuterated noralfentanil were crystallized as the hydrochloride salts and their final purity was determined by GC–MS, both as native compounds and as the pentafluoropropionyl derivatives. Both compounds were found to be >95% pure. The isotopic composition of deuterated NA (excluding natural isotopic abundances) was 0% d_0, 0% d_1, 0% d_2, 3% d_3, 25% d_4, and 72% d_5, and the compound was considered to be d_5-NA. AMX, AM5 and AM3 were synthesized by the reaction of an appropriate amine with the proper acid anhydride [10]. The deuterated analogues of AMX and AM5 were synthesized from d_5-aniline and 4-hydroxy-d_4-aniline, respectively. For deuterated AMX, the isotopic composition (excluding natural isotopic abundances) was found to be 1% d_0, 2% d_1, 3% d_2, 2% d_3, 3% d_4, and 88% d_5. For deuterated AM5 the isotopic composition was 4% d_0, 4% d_1, 12% d_2, 39% d_3, and 40% d_4. The deuterated compounds were considered to be d_5-AMX and d_4-AM5, respectively.

Stock solutions of NA.HCl and d_5-NA.HCl were prepared in methanol or 100 m*M* potassium phosphate buffer (pH 7.4) as appropriate. Stock solutions of AMX, AM5, AM3 and their deuterated analogues were prepared in methanol. All solutions were stored at 4°C.

2.2. Incubation conditions

Microsomes were prepared from human livers as described previously [4]. Metabolite formation was determined in 1 ml incubation mixtures containing 0.1 *M* potassium phosphate buffer (pH 7.4), 1 m*M* NADPH, human liver microsomes and ALF at 37°C. Microsomal protein and substrate concentrations are provided in the figure legends. Reactions were initiated after a 3 min preincubation period by the addition of NADPH, and were terminated with 0.3 ml of 0.5 N NaOH.

2.3. Analytical procedure

GC–MS analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced to an HP 5971 mass selective detector, using a DB-5 or DB-17 fused-silica capillary column (15 m × 0.32 mm I.D. × 0.25 μm film thickness) (J and W, Folsom, CA, USA). The injector, containing a quartz liner, was operated in the splitless mode. The helium flow-rate was 60 ml/min and the column head pressure was 5 p.s.i. (1 p.s.i. = 6894.76 Pa). Although both DB-5 and DB-17 columns provided adequate resolution, the DB-17 column provided slightly better peak shapes compared to the DB-5 and was used preferentially for quantitative analyses.

For determination of ALF metabolites in microsomal reaction mixtures, standard curves of peak area ratios (d_0/d_5 NA) vs. ng d_0 NA added (routinely 0–2000 ng/ml); (d_0/d_5-AMX) vs. ng d_0-AMX added (routinely 0–600 ng/ml); (d_0/d_4-AM5) vs. ng d_0-AM5 added (routinely 0–600 ng/ml); and (d_0-AM3/d_4-AM5) vs. ng d_0-AM3 added (routinely 0–600 ng/ml) were prepared using blank microsomes. Standard curves were used to quantify NA, AMX, AM5 and AM3 concentrations in unknown samples. Inter-day coefficients of variation for NA (2500 ng/ml, $n = 3$) and AMX (500 ng/ml, $n = 3$) were less than 5%.

For determination of NA, the internal stan-

dard d_5-NA (225 ng) was added to the basified mixture (pH 12–13) which was then extracted twice with 4 ml ethyl acetate by vortexing for 30 sec. The organic layers were pooled and dried with sodium sulfate. The ethyl acetate layer was transferred to a clean 13 × 100 ml culture tube, evaporated to dryness at 30–40°C under a nitrogen stream, and reconstituted in 110 μl ethyl acetate. The pentafluoropropionyl (PFP) derivatives of NA and d_5-NA were prepared by adding 40 μl pentafluoropropionic anhydride to the ethyl acetate solution and heating at 60°C for 30 min. The samples were again evaporated to dryness under a nitrogen stream, and dissolved in ethyl acetate (100 μl) for GC–MS analysis.

NA was quantified by selected-ion monitoring of NA-PFP (*m/z* 377) and d_5-NA-PFP (*m/z* 382). Optimum injector and transfer line temperatures for NA-PFP and d_5-NA-PFP, based on maximum peak areas, were found to be 290°C and 300°C, respectively. The oven temperature was held at 70°C for one min, increased at 16°C/min to 240°C, then increased at 70°C/min to 280°C, and maintained at 280°C for 5 min (unless otherwise indicated in figure legends).

For determination of AMX, AM5 and AM3, internal standards (200 ng each of d_5-AMX and d_4-AM5) were added to the basified reaction mixture, which was then rendered acidic (pH 1–2) by adding 350 μl of 1 *M* HCl. The samples were kept at room temperature for 30 min and extracted by vortexing with 2 × 4 ml ethyl acetate. The organic layers were pooled, dried with sodium sulfate, evaporated to dryness at 30–40°C under a nitrogen stream, and reconstituted in 100 μl ethyl acetate. Trimethylsilyl (TMS) derivatives were prepared by adding 50 μl BSTFA–1% TMCS to the ethyl acetate solution and heating at 65°C for 40 min. Samples were transferred to autosampler vials and analyzed by GC–MS without removing the excess derivatizing reagent.

AMX was quantified as the mono-TMS derivative, using d_5-AMX as the internal standard. AM5 and AM3 were quantified as their di-trimethylsilyl derivatives using d_4-AM5 as the internal standard for both compounds. The following ions were monitored in selected-ion mode: AMX-TMS (*m/z* 221), AM5-diTMS (*m/z* 294), AM3-diTMS (*m/z* 280) and the internal standards d_5-AMX-TMS (*m/z* 226) and d_4-AM5-diTMS (*m/z* 298). Optimum injector and transfer line temperatures for AMX-TMS, AM5-diTMS and AM3-diTMS, based on maximum peak areas, were 190°C and 290°C respectively. The column oven temperature was held at 50°C for one min, raised at 15°C/min to 200°C, then increased at 70°C/min to 280°C (unless indicated otherwise).

3. Results

For the development of this assay, attention was focussed on the major ALF metabolites, NA, AMX, AM5 and AM3. Although small amounts of AM6 have been reported previously in vivo [6], this metabolite was not included in the development of the current assay because of its reported chemical instability [9] and its presence only as a minor, secondary metabolite.

3.1. Metabolite derivatization

Derivatization of all ALF metabolites was required to improve peak broadening and tailing, and to increase the ion mass used for quantitation. NA was efficiently acylated with PFAA to pentafluoropropionylated NA (NA-PFP). Two prominent ions at *m/z* 377 ($M^+ - CH_2OCH_3$) and *m/z* 321 ($377 - COCH_2CH_3$) were identified, although the parent ion (*m/z* 422) was not observed (Fig. 2A). For d_5-NA-PFP, corresponding major fragments at *m/z* 382 ($M^+ - CH_2OCH_3$) and *m/z* 326 ($382 - COCH_2CH_3$) were identified. NA was detected and quantitated as NA-PFP using selected-ion monitoring at *m/z* 377, which provided fewer ion interferences than at *m/z* 321[1].

[1] NA was also efficiently trifluoroacylated with MBTFA. Major fragment ions at *m/z* 327 ($M^+ - CH_2OCH_3$) and *m/z* 271 ($327 - COCH_2CH_3$) were identified, while the parent ion (*m/z* 372) was not observed. PFAA was used for NA derivatization, however, because of the higher mass fragments obtained.

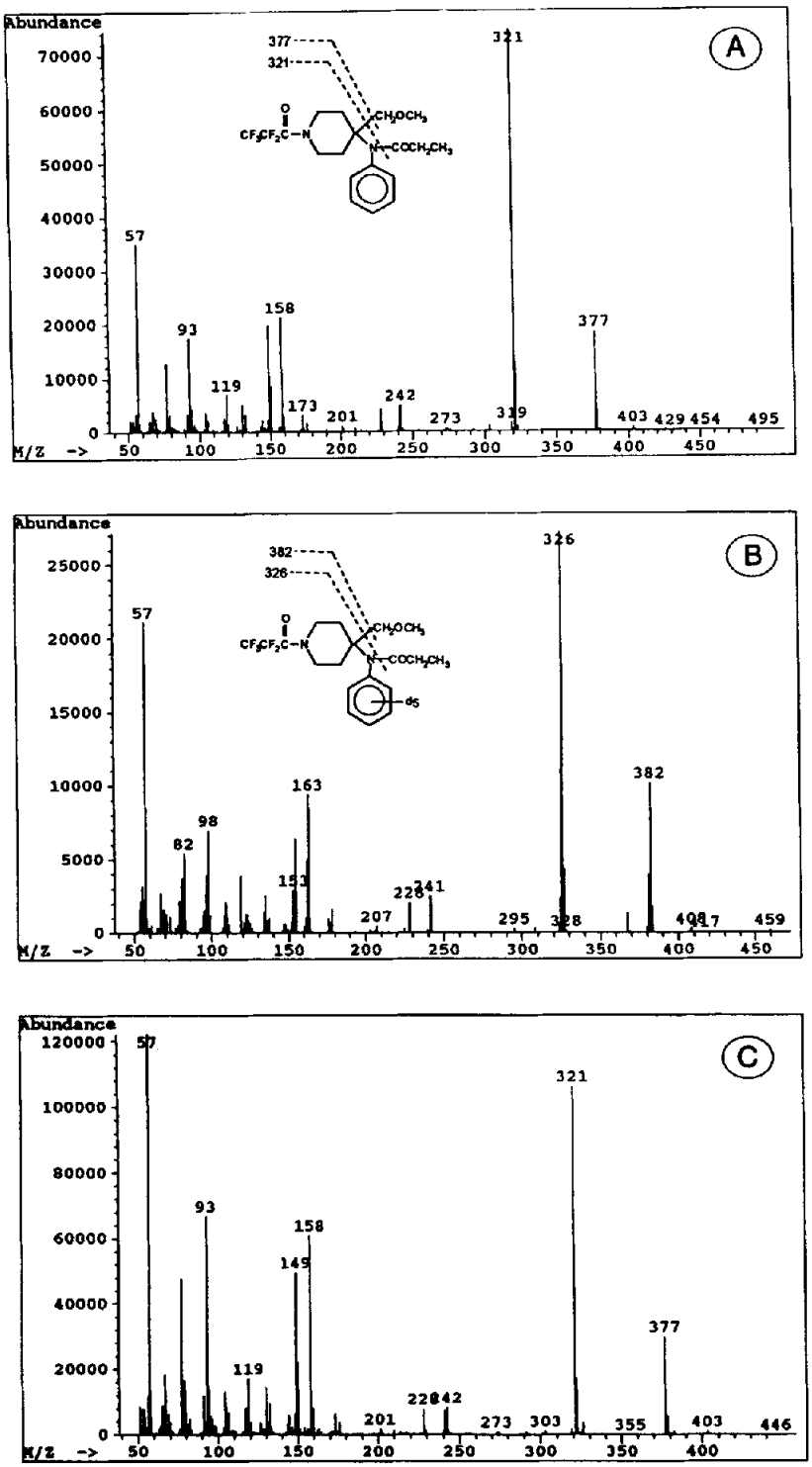

Fig. 2. GC–MS analysis of NA. (A) Mass spectrum of the pentafluoropropionyl derivative of noralfentanil (NA-PFP; retention time 14.8 min). (B) Mass spectrum of d_5-NA-PFP. (C) GC–MS analysis of the PFP derivative of noralfentanil resulting from alfentanil biotransformation. Alfentanil (100 μM) was incubated with human liver microsomes (0.5 mg/ml) and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) for 40 min, and metabolites were extracted and derivatized as described in the Experimental section.

Derivatization of AMX, AM5 and AM3 was complex. PFAA acylation was unsatisfactory because AMX remained mostly unreacted while AM5 and AM3 yielded primarily mono-PFP (phenolic) derivatives, with small and highly variable amounts of di-PFP derivatives. No reaction conditions were identified that would consistently yield either exclusively mono-PFP or di-PFP derivatives of AM5 or AM3. Reproducible, quantitative derivatization of AMX, AM5 and AM3 was achieved by silylation with BSTFA–1% TMCS, producing the monoTMS derivative of AMX and the diTMS derivatives of AM5 and AM3[2]. The mass spectrum of AMX-TMS showed a molecular ion at m/z 221, a prominent $M^+ - 15$ ion at m/z 206 (loss of a TMS methyl group), and a major m/z 132 fragment $[M^+ - OSi(CH_3)_3]$ (Fig. 3A). The d_5-AMX-TMS analog showed a corresponding molecular ion at m/z 226 and a $M^+ - 15$ ion at m/z 211. AM5-diTMS (Fig. 4A) and AM3-diTMS (Fig. 5) gave abundant molecular ions at m/z 309 and m/z 295, respectively, with a molecular ion at m/z 313 for d_4-AM5-diTMS. Ions observed were again due to the loss of methyl and O-TMS fragments. For quantitation using selected-ion monitoring, the m/z 221, 294 and 280 ions for AMX-TMS, AM5-diTMS, and AM3-diTMS, respectively, were observed[3].

Since PFAA effectively derivatized NA and the phenolic groups of AM5 and AM3, and silylation derivatized the amide group of AMX, AM5, and AM3, dual derivatization was explored. Metabolites (NA, AMX, AM5 and AM3) were derivatized with PFAA and the excess anhydride evaporated, forming NA-PFP, AM5 and AM3 O-PFP derivatives, and unreacted AMX. BSTFA–TMCS was then added, with the goal of trimethylsilylating the amide group of AMX, AM5-PFP and AM3-PFP. After

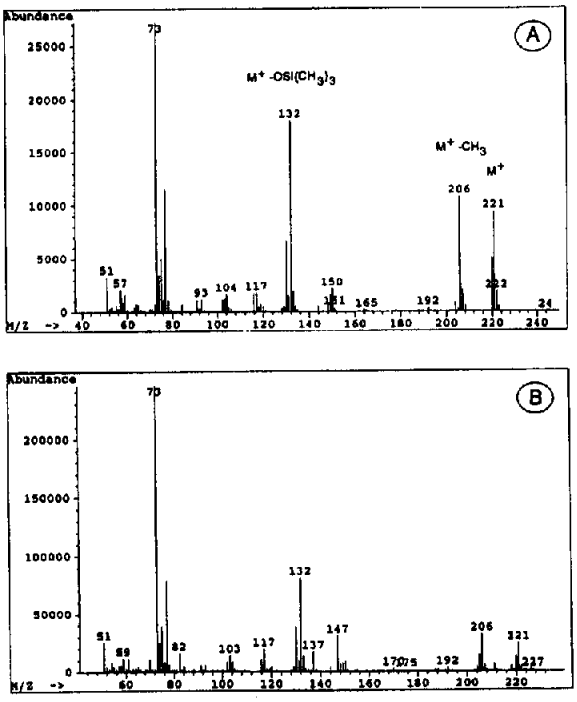

Fig. 3. GC–MS analysis of AMX. GC conditions included a 15-m DB-5 column, injector and transfer line temperatures of 270°C and 290°C, and an oven program from 100°C to 280°C at 10°C/min. (A) Mass spectrum of AMX-TMS (retention time 6.6 min). (B) GC–MS analysis of the TMS derivative of AMX resulting from alfentanil biotransformation by human liver microsomes. Incubation conditions are described in the legend to Fig. 2.

evaporating excess BSTFA–TMCS, NA-PFP, AMX, AM5-monoTMS and AM3-monoTMS were identified, indicating conversion of AM5 and AM3 from O-PFP to O-TMS derivatives. No amide TMS derivatives of AMX, AM5 or AM3 were observed. Without removal of excess BSTFA–TMCS, however, AMX-TMS, AM5-diTMS, and AM3-diTMS were observed and NA-PFP appeared quite resistant to breakdown by BSTFA–TMCS, in contrast to parent NA.

These results indicated that the optimal methods for derivatization involved acylation of the piperidine nitrogen of NA, and silylation of both the amide and hydroxyl groups of AMX, AM5 and AM3. Therefore, NA was analyzed routinely as the PFP derivative. AMX was analyzed as the TMS derivative and AM5 and AM3 were both analyzed as the diTMS derivatives.

[2] Derivatization with BSTFA alone resulted in a mixture of unreacted AMX and AMX-TMS, and mono- and di-TMS derivatives of AM5 and AM3.

[3] Attempted NA silylation with BSTFA–TMCS was unsuccessful, yielding primarily unreacted NA with partial degradation of NA to AMX-TMS. No peak corresponding to a TMS derivative of NA was observed.

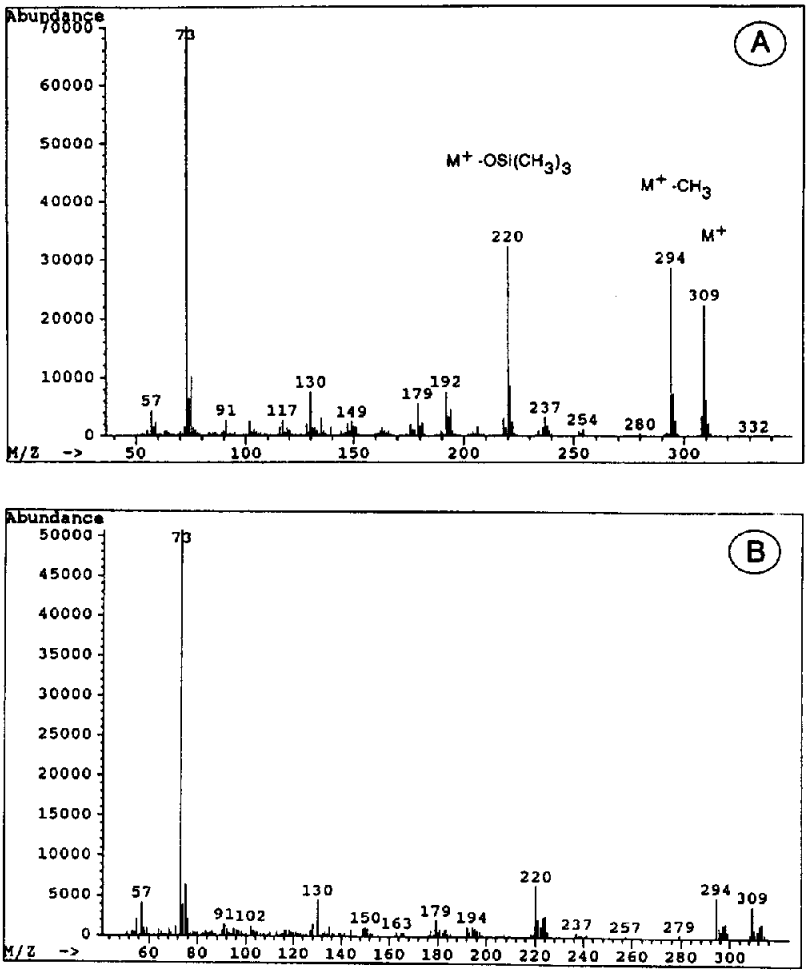

Fig. 4. GC–MS analysis of AM5. GC conditions are identical to those described in Fig. 3. (A) Mass spectrum of AM5-diTMS (retention time 9.5 min) prepared using BSTFA with 1% TMCS. The mono-TMS derivative of AM5 (11.1 min) was not observed under these conditions. (B) GC–MS analysis of the diTMS derivative of AM5 resulting from alfentanil biotransformation by human liver microsomes. Incubation conditions are described in the legend to Fig. 2.

3.2. Metabolite extraction

The disparate structures and functionalities of the various ALF metabolites prevented the development of a single extraction scheme for all compounds of interest. Rather, NA was extracted using a different set of conditions than those used for AMX, AM5 and AM3.

NA was extracted under basic conditions (pH ~ 12). Ethyl acetate was found to provide the highest NA recovery (90%) under these conditions. Other solvent systems tested were ethyl acetate–diethyl ether (2:1, v/v) in the absence and presence of saturated sodium chloride, heptane–isoamyl alcohol (98.5:1.5, v/v), and pentane–isoamyl alcohol (98.5:1.5, v/v). Standard curves for NA quantitation in microsomes were linear over the range of 0–10,000 ng/ml (Fig. 6A). The limit of NA quantification was 5 ng/ml at 4:1 signal to noise. For routine analysis NA standard curves were prepared over the range 0–2000 ng/ml, with excellent linearity observed ($r^2 = 0.99$).

AM3 has been reported to be unstable in

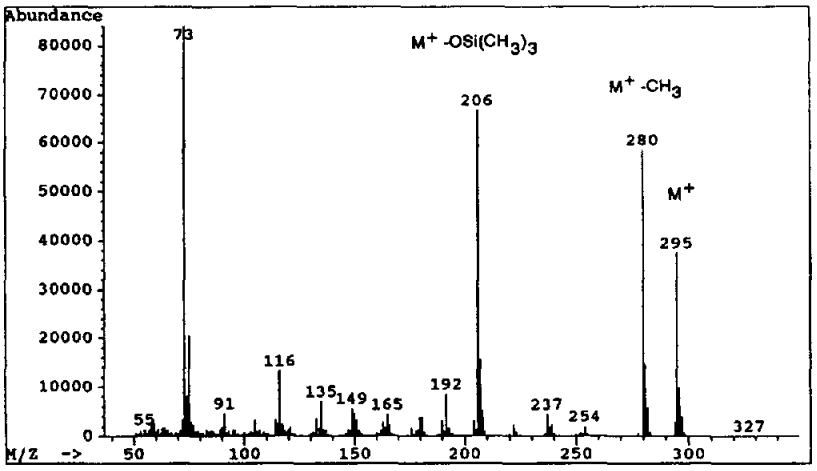

Fig. 5. GC–MS analysis of AM3. Shown is the mass spectrum of AM3-diTMS (retention time 10.3 min). GC conditions are identical to those described in Fig. 3.

strongly basic solutions [10]. Therefore AMX, AM5 and AM3 were extracted at neutral pH [11], with optimal extraction efficiency observed with ethyl acetate. Metabolite recovery was 70%, 90% and 85%, respectively, for AMX, AM5 and AM3 using ethyl acetate at neutral pH. Later experiments, however, indicated that small amounts of both ALF and NA could be extracted into ethyl acetate at neutral pH and contributed to the AMX-TMS peak on GC–MS, most likely by partial breakdown of ALF and/or NA by BSTFA and formation of AMX-TMS, as described previously. To minimize extraction of ALF and NA from microsomal incubation samples into the organic layer and hence contribution to apparent AMX-TMS quantitation, AMX, AM5 and AM3 were subsequently extracted under acidic conditions (pH 1–2). No deuterium exchange occurred with either d_5-AMX or d_4-AM5 for several hours at this acidic pH. Standard curves for AMX (Fig. 6B), AM5 (Fig. 6C) and AM3 (not shown) quantitation in microsomes were linear over the range of 0–10,000 ng/ml. Limits of quantification for AMX and AM5 were 15 ng/ml and 5 ng/ml, respectively. For routine analysis AMX standard curves were prepared over the range 0–600 ng/ml, with excellent linearity observed ($r^2 = 0.99$).

3.3. Microsomal incubations

The above methods of extraction and GC–MS analysis were applied to the detection and quantitation of metabolites formed by ALF incubation with human liver microsomes. Following extraction and derivatization, the formation of NA (Fig. 2C), AMX (Fig. 3B) and AM5 (Fig. 4B) was identified by comparing the mass spectra of unknown peaks with those of authentic standards having the same retention times. Formation of AM3 was not observed. The time-dependent formation of NA, AMX and AM5 was followed by selected-ion monitoring of aliquots removed from the reaction mixture (Fig. 7), as outlined in the Experimental section.

4. Discussion

Two schemes, utilizing acylating and silylating agents for the different metabolite functional groups, were required for optimal derivatization of all the ALF metabolites of interest. PFAA efficiently derivatized noralfentanil to N-pentafluoropropionyl noralfentanil (NA-PFP), but was not suitable for AMX, AM5 and AM3 due to varying reactivity of the amide functionality.

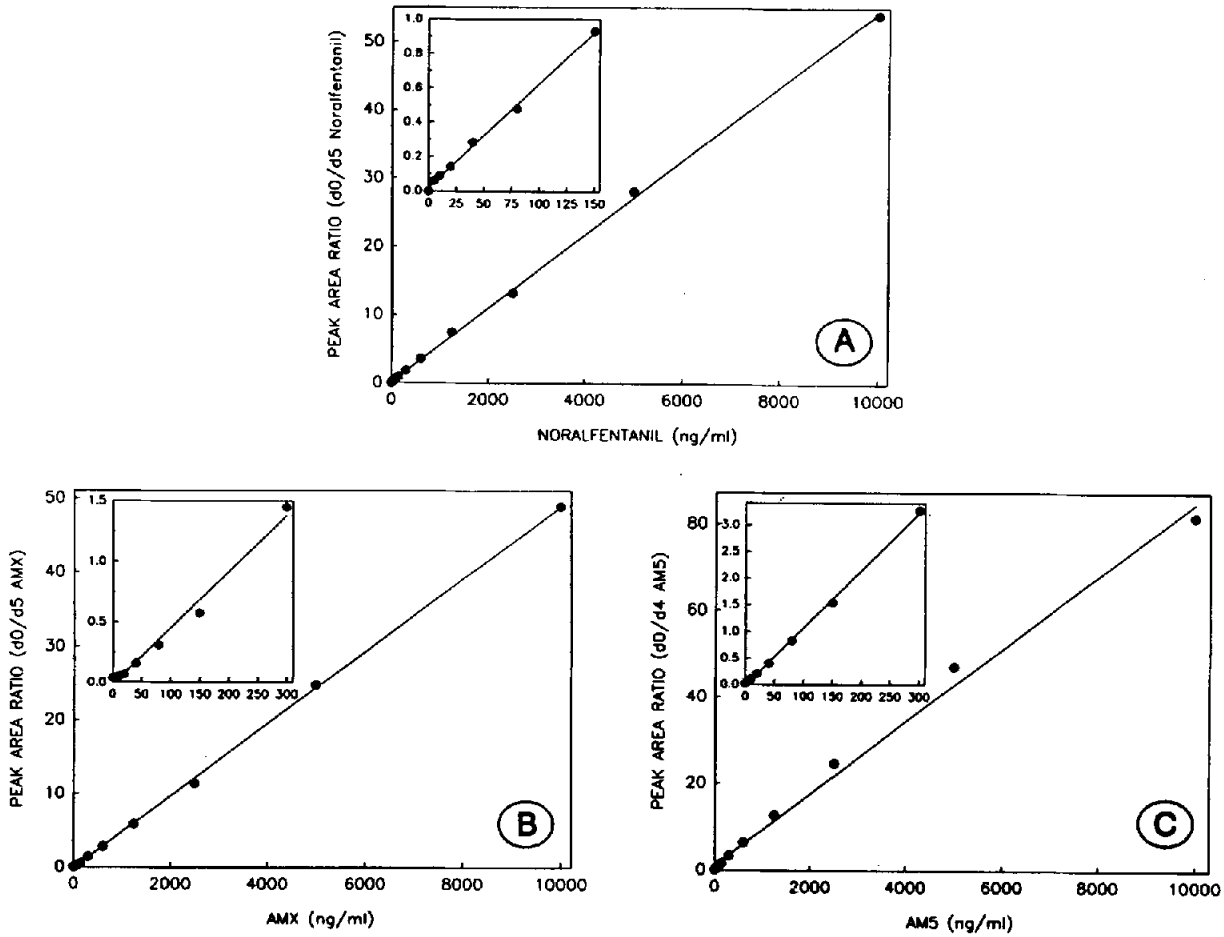

Fig. 6. Standard curves for GC–MS analysis of alfentanil metabolites in human liver microsomes. Curves are not corrected for minor contributions of unlabelled metabolite (0–4%) in the deuterated standards. (A) Noralfentanil. Peak area ratios for *m/z* 377 and 382 were used for d_0- and d_5-NA-PFP ($r^2 = 0.99$). The inset magnifies the region for 0–150 ng/ml NA. (B) AMX. Peak area ratios for *m/z* 221 and 226 were used for d_0- and d_5-AMX-TMS ($r^2 = 0.99$). The inset magnifies the region for 0–300 ng/ml AMX. (C) AM5. Peak area ratios for *m/z* 294 and 298 were used for d_0- and d_4-AM5-diTMS ($r^2 = 0.99$). The inset magnifies the region for 0–300 ng/ml AM5.

Analysis of AMX, AM5 and AM3 required formation of trimethylsilyl derivatives. TMCS addition to BSTFA was required to ensure complete derivatization of all metabolites. The amide-TMS group was moisture sensitive, particularly compared to the phenolic-TMS group, hence samples were analyzed without removing the unreacted BSTFA prior to GC–MS analysis.

Analysis of human liver microsomal incubations with ALF demonstrated the application of the GC–MS assay. NA was the major in vitro metabolite, with AMX formed at one fourth to one half the rate of NA, in agreement with previous observations [7]. In addition to these two metabolites, we were able to identify N-(4-hydroxyphenyl)propanamide (AM5) as a minor in vitro metabolite. AM5 formation in vitro was not detected in a previous investigation of human liver microsomal ALF metabolism, using radio-HPLC [7]. Thus, using the GC–MS assay,

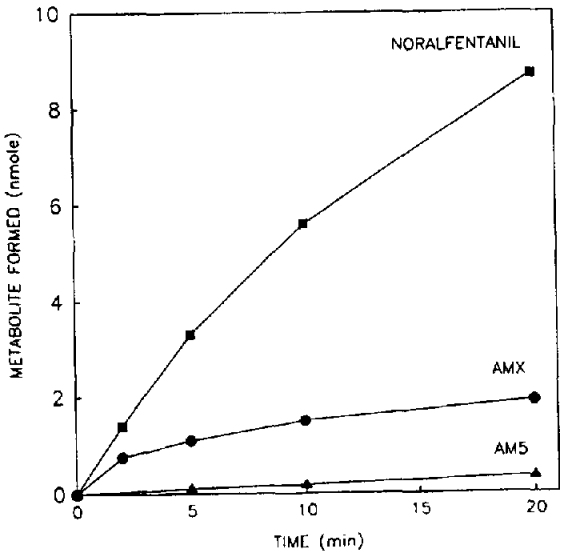

Fig. 7. Human liver microsomal alfentanil metabolism in vitro. Reaction mixtures contained 0.5 mg/ml microsomal protein, 20 μM alfentanil, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4). At the indicated times, aliquots were removed, added to NaOH and analyzed as described in Experimental using GC–MS with selected-ion monitoring.

human liver microsomal ALF metabolism in vitro is a more representative model for human ALF metabolism in vivo than using the HPLC assay. AM3, present as a minor metabolite in clinical investigations of ALF disposition [6], was not detected in our in vitro microsomal incubations. This result was expected, since AM3 formation from AMX or AM5 requires N-acyltransferase, which is not a microsomal enzyme.

In summary, we describe a sensitive GC–MS assay for the detection and quantitation of alfentanil metabolites. Compared with previously employed radio-HPLC assays, the GC–MS assay obviates the need for radiolabelled ALF and permits the detection and quantitation of several metabolites. The GC–MS assay has been applied successfully to in vitro studies of human alfentanil metabolism.

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