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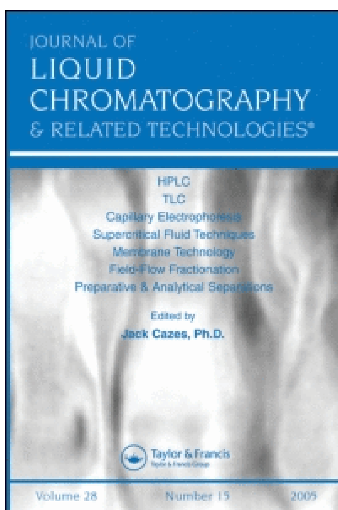

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DIACETYLMORPHINE AND ITS METABOLITES IN PLASMA BY HPLC WITH DIODE-ARRAY AND ATMOSPHERIC PRESSURE IONIZATION MASS SPECTROMETRIC DETECTION

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ABSTRACT

Based on reversed phase high performance liquid chromatography with gradient elution and diode-array detection (HPLC-DAD) a specific and sensitive assay for the quantitation of diacetylmorphine (heroin, DAM) and its primary and secondary metabolites 6-acetylmorphine, morphine, morphine-3-β-D-glucuronide, morphine-6-β-D-glucuronide and nor-morphine in plasma was developed. Confirmation of the peak assignment was performed by atmospheric pressure ionization-mass spectrometry (API-MS). Plasma samples were extracted on C_{18} ODS-2 columns using an automated solid-phase extraction (SPE) unit. The recovery was between 45 and 68%.

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The quantitation limit of DAM and its metabolites was 50 and 10 ng/mL, respectively. The inter-day precision was in the range of 4.0 to 17.6%. The described method was applied for pharmacokinetic studies which were part of the evaluation and validation of DAM preparations used within a heroin-assisted treatment program for dependent drug users.

INTRODUCTION

In 1994 the Swiss Federal Office for Public Health (SOPH) initiated heroin-assisted treatment programs for dependent drug users, i.e. the medical prescription of diacetylmorphine (heroin, DAM) to heavy heroin addicts under strictly controlled clinical conditions. Supporting pharmaceutical research was mainly focussed on the pharmacokinetic and pharmacodynamic evaluation and validation of different application forms (intravenous, rectal, oral, pulmonal) of high-dosed DAM. The HPLC-DAD method used at the beginning of the project for plasma profiling showed limited specificity and sensitivity, was sensitive to matrix interferences, and not suited for mass analysis.[1] Other HPLC methods, basing on fluorimetric,[2-5] electrochemical,[6-8] ultraviolet,[9,10] or diode-array detection[11,12] or combination of these techniques,[13-16] were developed only for the analysis of morphine (M) and its pharmacologically active metabolites morphine-3-β-D-glucuronide (M3G) and morphine-6-β-D-glucuronide (M6G). Recently, HPLC-MS with electrospray,[17,18] atmospheric pressure (API),[17,19] or atmospheric pressure chemical ionization (APCI)[17,20] interfaces has been introduced for opiate plasma analysis. The aim of the present work was to establish a highly specific HPLC-DAD-MS method with automated sample preparation which should allow the quantitative serial monitoring of the complex metabolic pattern of DAM in plasma including 6-acetylmorphine (6AM), (M), M3G, M6G and normorphine (NM).

EXPERIMENTAL

Chemicals and Standards

Ethylmorphine hydrochloride (EM, IS; Merck, Basel, Switzerland); DAM (Sanofi Chimie, Gentilly Cedex, France) and M (Hänseler, Basel, Switzerland), both provided as hydrochloride; 6AM and NM, both synthesized as hydrochloride at the Institute of Pharmacy, University of Bern, Switzerland; M3G (Sigma, Buchs, Switzerland); M6G (Lipomed, Arlesheim, Switzerland). The identity and purity of all substances were checked by HPLC-DAD and/or GC-MS. All other chemicals and reagents were of analytical grade or HPLC quality and supplied by Merck (Basel, Switzerland).

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Instrumentation

The HPLC system consisted of a Hewlett-Packard (HP) 1090 Series II liquid chromatograph (Hewlett-Packard, Waldbronn, Germany), a HP 1090L autosampler, a HP 1040M diode-array detector, and a 3DChemstation (Revision A.02.05). For validation of the HPLC-DAD procedure a Hewlett-Packard (HP) 1100 Series LC/MSD system (Hewlett-Packard, Waldbronn, Germany) with 3D ChemStation Rev. A.05.01 Software (1997, Windows NT) was used.

An ASPEC (Automatic Sample Preparation with Extraction Columns) system equipped with a Dilutor 401 (Gilson, Villiers Le Bel, France) was used for the automated solid phase extraction (SPE) of plasma samples.

Chromatographic Conditions

The separation of the plasma extracts was performed at 45°C on a 125 x 2 mm i.d. column and a 8 x 3 mm i.d. precolumn, both packed with Nucleosil-120 C-8 and a particle size of 3 µm (Macherey-Nagel, Oensingen, Switzerland) by the following multi-step gradient: 0-1 min, 3.5% B, isocratic; 1-3 min, 3.5-13% B, linear; 3-4 min, 13-15% B, linear; 4-20 min, 15% B, isocratic; 20-21 min, 15-100% B, linear; 21-24 min, 100% B, isocratic; 24-25 min, 100-3.5% B, linear. A: bidistilled water, containing 0.05% (v/v) trifluoro acetic acid (TFA), B: acetonitrile, containing 0.05% (v/v) TFA.

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The reconditioning time was 15 min. The flow rate was set at 330 µL/min. Detection and quantitation were at 210 nm. Online DAD spectra were recorded from 192 to 350 nm and used for peak purity checks. Peak assignment was achieved by library match of the recorded UV spectra and by recording the molecular masses m/z 272 for [NM^+-H]; m/z 462 for [$M3G^+$-H] and [$M6G^+$-H]; m/z 286 for [M^+-H]; m/z 328 for [$6AM^+$-H]; m/z 314 for [EM^+-H] and m/z 370 for [DAM^+-H].

Calibration

The quantitation of DAM and its metabolites was performed on the basis of peak areas using the internal standard method. Data for the calibration graphs were recorded with aqueous solutions of pure standards at known concentrations. Each calibration graph was calculated by linear regression analysis from triplicate determinations at the low, medium, and high concentration level of 10, 100, and 1000 ng/mL, respectively. To match the levels found in real samples the additional calibrators 500 (NM), 3000 (M6G, M), 4000 (DAM), 6000 (M3G), and 7000 ng/mL (6AM) were used.

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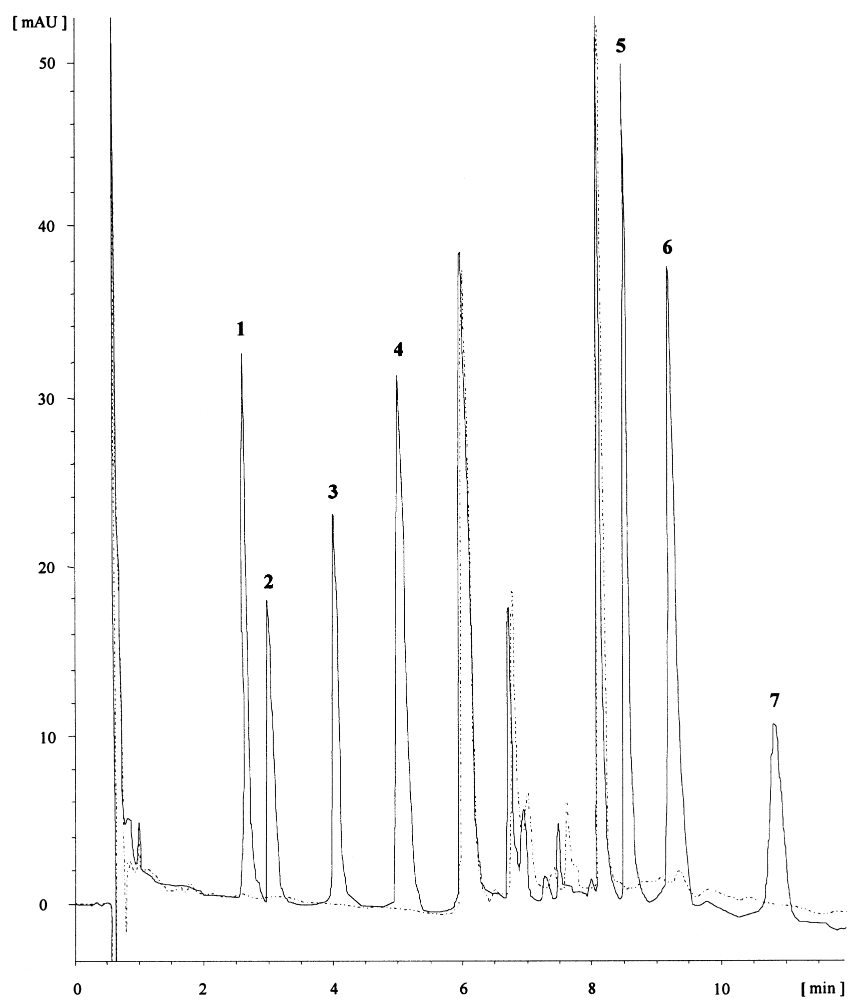

Figure 1. HPLC-DAD profiles of blank (----) and spiked plasma (-). Legend: (1) M3G; (2) NM; (3) M6G; (4) M; (5) 6AM; (6) IS; (7) DAM.

Extraction Procedure

The extraction of plasma samples was performed on C-18 ec, 3 mL, 500 mg SPE columns (Macherey-Nagel, Oensingen, Switzerland) by using a robot system. Conditioning was by 10 mL of methanol, followed by 5 mL of acetonitrile - 0.01 M phosphate buffer (pH 2.1) 40:60 (v/v) and 10 mL of

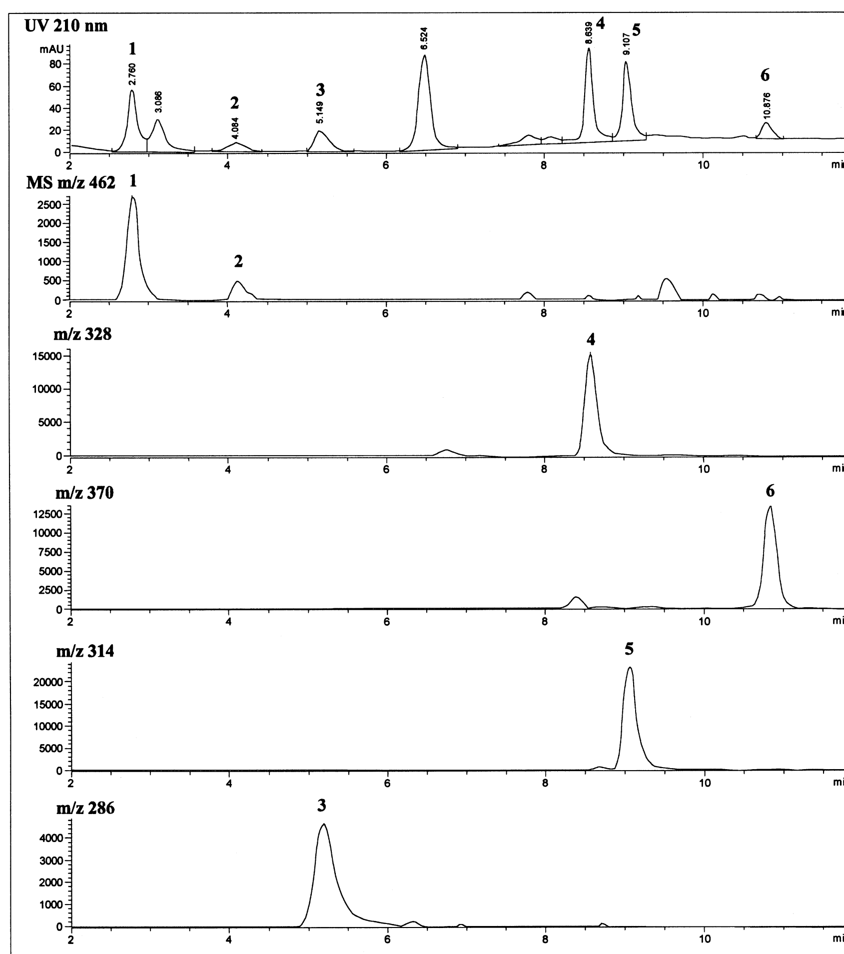

Figure 2. HPLC-DAD-API-MS plasma profile 6 min after the intravenous injection of 200 mg DAM. Legend: see Fig. 1.

bidistilled water. To 1.5 mL of plasma, 4.5 mL of a 0.5 M carbonate buffer (pH 9.3) and 150 μL of the IS solution (0.001% EM in water) were added and vortexed for 10 sec. 4.1 mL (aliquot of 1 mL plasma) of this solution was applied on the SPE column. Washing was done by 20 mL of 0.005 M carbonate buffer (pH 9.3) followed by 0.5 mL of bidistilled water and 0.35 mL of

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Table 1

Validation Data

Analyte	Recovery (%, n=6)	Inter-Day Precision (RSD, %, n=6) at 100/1000/* ng/mL	Limit of Quantitation (ng/mL)
DAM	44.8	16.5/14.3/12.2	50
6AM	59.2	6.3/7.7/10.1	10
M	66.5	14.8/7.4/4.0	10
NM	63.8	17.6/10.1/12.8	10
M6G	50.1	12.0/11.8/12.8	10
M3G	53.5	9.2/10.5/4.9	10
IS	67.7	------	10

* 4000 (DAM), 7000 (6AM), 3000 (M, M6G), 500 (NM), 6000 (M3G) ng/mL.

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acetonitrile-0.01 M phosphate buffer (pH 2.1) 40:60 (v/v). Elution of the analytes was by two portions of 0.6 mL of acetonitrile-0.01 M phosphate buffer (pH 2.1) 40:60 (v/v). The eluate was evaporated to dryness under nitrogen, reconstituted in 100 µL of bidistilled water and a 10 µL-aliquot injected into the HPLC-DAD.

Precision

For every concentration level six blank plasma samples were spiked with the analytes. These samples were then extracted as described above and analyzed at different days within a month using the present method.

Sample Collection

Blood specimens (9 mL) from 5 participants of the SOPH heroin-assisted treatment programs were obtained through a peripheral vein catheter and collected in heparinized tubes. Samples were drawn 10 min before (baseline) and 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, and 120 min after the intravenous injection of 200 mg DAM.

After centrifugation (2500 g, 10 min) the plasma samples were stored at -25°C in two polypropylene tubes containing sodium fluoride (20 mg/mL plasma).

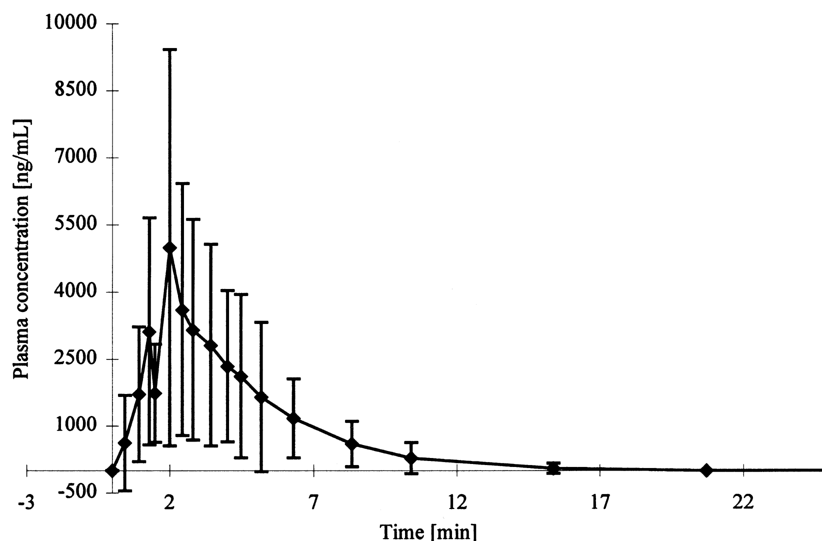

Figure 3. Plasma concentration-time profiles of DAM (mean ± SD, n = 5, 200 mg DAM i. v.).

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RESULTS

Sample Preparation

Figure 1 shows that with reasonable recoveries of the analytes (Table 1) most of the plasma matrix can be eliminated by extraction on reversed phase SPE columns. The robotic SPE workstation allows automated conditioning, washing, and elution and is, with a capacity of 35 samples per 24 h, suited for serial analysis. To prevent in vitro enzymatic desacetylation of DAM and 6AM sodium fluoride is added as soon as possible to the collected plasma specimens. At -20°C the plasma samples and extracts are stable for several months.

HPLC System

HPLC procedures based on C_{18} reversed phase materials with o-phosphoric acid, acetonitrile, and hexylamine as mobile phase have shown good selectivity and reproducibility for the determination of basic compounds differing strongly in their physicochemical characteristics. They are routinely used in our lab for the analysis of psychoactive compounds in pharmaceutical preparations[21] and biological matrices (body fluids, plant materials).[22-27] However, this type of

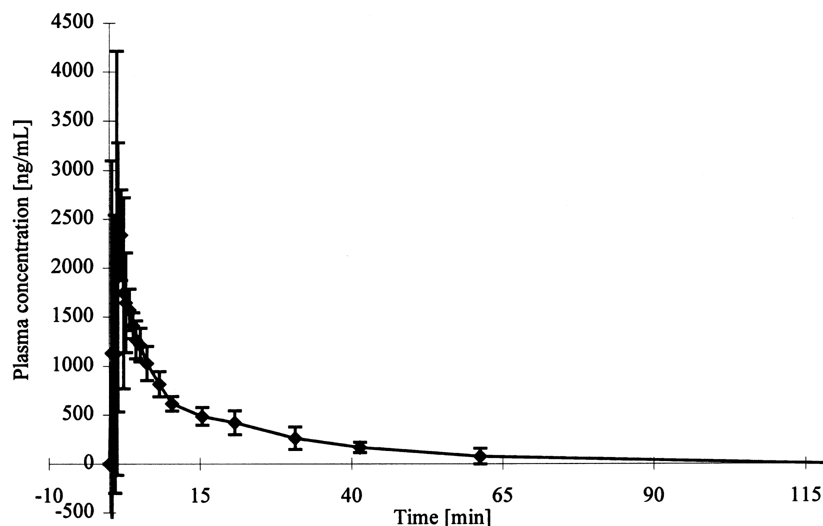

Figure 4. Plasma concentration-time profiles of 6AM (mean ± SD, n = 5, 200 mg DAM i. v.).

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mobile phase is not compatible with particle beam (PB) or atmospheric pressure (API) HPLC-MS interfaces. Therefore a new chromatographic system had to be evaluated. Experiments with C8 reversed phase materials and water-acetonitrile mixtures containing 0.01% (v/v) TFA showed a good resolution and a nice separation of the analytes of interest (Figure 1 and 2). Peak assignment was achieved using the retention time of standards, the corresponding DAD-UV spectra and the MS of the protonated compounds. The low UV cut-off of the mobile phase allowed the detection at 210 nm where most of the opiates exhibit a UV maximum. Calibration curves were linear in the range of 10 to 6000 ng/mL, and the regression equation showed correlation coefficients $r \geq 0.999$. The inter-day reproducibility was in the range of 4.0 to 17.6%. At a signal-to-noise ratio between 4.5 (DAM) and 15 (M, M3G) the limits of detection and quantitation were 50 ng/mL for DAM and 10 ng/mL for the metabolites. The validation data are summarized in Table 1.

Pharmacokinetic Application

The described method is extensively used for pharmacokinetic studies in heroin addicts participating in the SFOPH heroin-assisted treatment programs. Plasma-concentration time curves (mean ± S.D.) of DAM and its active metabolites 6AM, M and M6G (M3G not shown) after the intravenous

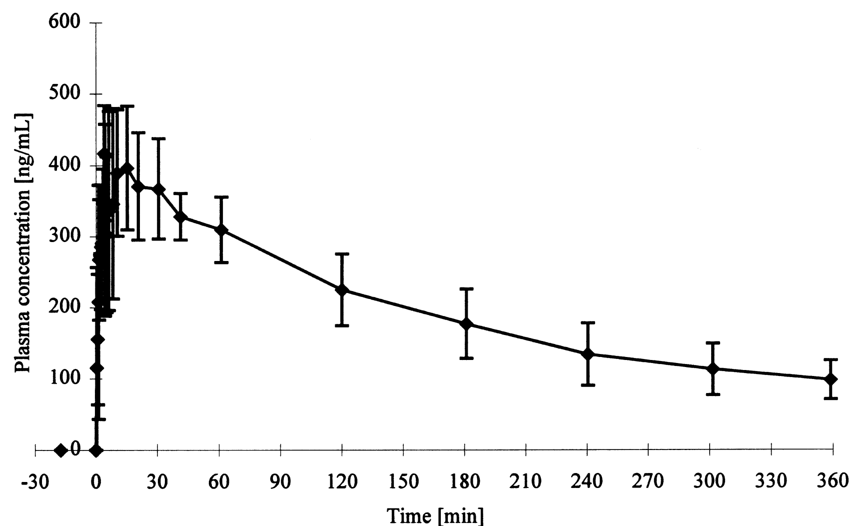

Figure 5. Plasma concentration-time profiles of M (mean ± SD, n = 5, 200 mg DAM i. v.).

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administration of 200 mg DAM to 5 subjects are shown in Figure 3-6. The interindividually very variable peak plasma levels of DAM (C_{max} 3380 ± 2737 ng/mL) and 6AM (C_{max} 2670 ± 1309 ng/mL) appeared already 2.5 ± 1.5 min and 2.5 ± 1.6 min after injection, respectively. This demonstrates the extremely rapid desacetylation of DAM ($t_{1/2\beta}$ 2.3 ± 0.3 min). 6AM was eliminated from the plasma within 60-125 min (DAM ($t_{1/2\beta}$ 23.4 ± 13.4 min) by metabolization to M. The highest concentrations (C_{max} 447 ± 79.5 ng/mL) of M were measured 7.0 ± 7.6 min after injection, decreasing to about 50% within 150 min.

By glucuronidation M was further metabolized to M6G and M3G, the dominating metabolite of DAM in plasma. Characteristic for M glucuronides was the slowly dropping curve of M6G after reaching the maximum plasma levels of 751 ± 168 ng/mL at 64.5 ± 31.0 min. NM could not be detected. We assume, that as like M, this minor metabolite is present mainly in the form of glucuronides.

DISCUSSION

The efficiency of the described HPLC-DAD procedure combined with automated sample preparation has been proven by the successful pharmacokinetic profiling of DAM and its polar and nonpolar metabolites in

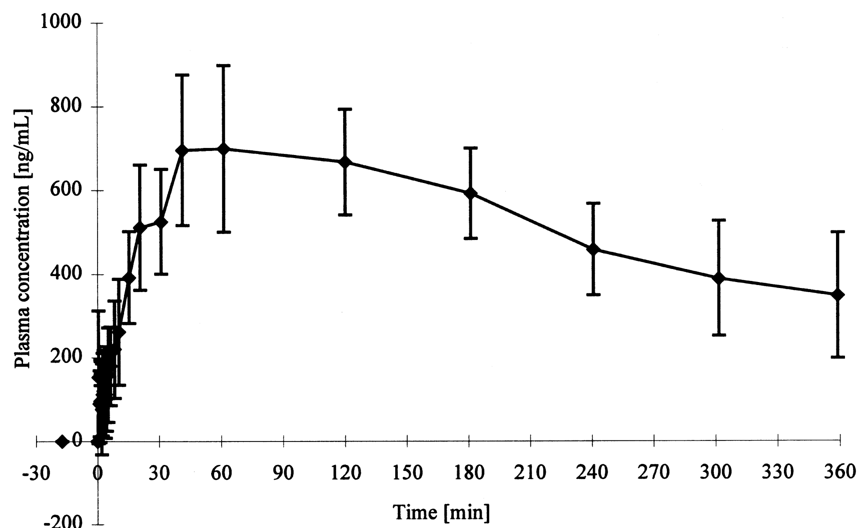

Figure 6. Plasma concentration-time profiles of M6G (mean ± SD, n = 5, 200 mg DAM i. v.).

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hundreds of plasma samples. As with 200 mg, very high intravenous DAM doses were administered and the sensitivity of DAD was, by far, sufficient for quantitation. After method development and validation the routine use of the costly MS detection in addition to DAD was no longer necessary. A check of the HPLC-DAD runs by HPLC-MS was later only required in rare cases of matrix interferences resulting in uncertain peak assignment.

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