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Short communication

High-performance liquid chromatographic monitoring of intravenously administered diacetylmorphine and morphine and their metabolites in human plasma

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Abstract

A rapid and selective reversed-phase high-performance liquid chromatographic assay with gradient elution and diode-array detection for diacetylmorphine, morphine, codeine, and their free and glucuronidated metabolites in plasma, was developed. After addition of ethylmorphine as internal standard the plasma samples were extracted using C_{18} ODS-2 solid-phase columns with a recovery better than 80%. The limit of quantitation using an injection volume of 2 μl was 25 ng/ml for each compound. The intra- and inter-day precision was better than 5%. The described method cannot only be used for pharmacokinetic studies but also for intoxication cases to monitor a wide range of opiates.

Keywords: Heroin; Diacetylmorphine; Morphine; Codeine

1. Introduction

As part of a 3-years project, the Swiss Federal Office of Public Health started, in 1994, with dispensing strictly medicinally-controlled 3,6-*O*-diacetylmorphine (DAM, heroin) to heavy heroin addicts ("Heroin Maintenance Programs"). Supporting pharmaceutical research is mainly focused on pharmacokinetic and pharmacodynamic aspects of different application forms (intravenous, oral, pulmonal etc.) of high-dosed DAM.

To establish pharmacokinetic profiles of the different application forms, a method using high-performance liquid chromatography with diode-array detection (HPLC–DAD) for the quantitative determination of DAM, morphine (M) and their metabolites 6-*O*-acetyl-morphine (6AM), normorphine (NM), morphine-3-*O*-glucuronide (M3G) and morphine-6-*O*-glucuronide (M6G) should be developed for plasma samples. The same method should also help to study a case of clinical intoxication with M.

Since the morphine glucuronides are known to be pharmacologically active [1], the interest for sensitive quantitation methods has increased. Most of the published procedures use high-performance liquid chromatography (HPLC) with fluorescence [2–6], electrochemical [5,7–9], UV or diode-array detection (DAD) [10–13]. All these methods were mainly developed for the detection of M, M3G, M6G and therefore were not suited to our purposes. To the best

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of our knowledge, up to now, no methods exist which allow the quantitation of DAM and its main metabolites within one extraction procedure and one HPLC run. Some experiments with capillary zone electrophoresis were performed earlier [14] but the lack of sensitivity was one of the reasons to evaluate another technique for the present work.

2. Experimental

2.1. Chemicals and standards

M, codeine (C), ethylmorphine (EM, I.S.), NM, M3G, M6G and codeine-6-*O*-glucuronide (C6G) were obtained as hydrochlorides through Sigma (Fluka, Buchs, Switzerland). Pharmaceutically pure DAM hydrochloride (purity >98%) was obtained by the Swiss Federal Office of Public Health (Bern, Switzerland). Following the methods of Wright [15] and Welsh [16] 6AM and 3-*O*-acetylmorphine (3AM) were prepared by hydrolysis of DAM and acetylation of M, respectively. Dihydromorphine (DHM) was obtained by reduction of M on palladium under hydrogen atmosphere, using the procedure described by ElSohly et al. [17]. The purity of all substances was checked by HPLC–DAD and gas chromatography–mass spectrometry (GC–MS) and was >98% for each compound. All chemicals were of analytical grade or HPLC quality and supplied by Merck (Basel, Switzerland) or Fluka (Buchs, Switzerland).

2.2. Instrumentation

The HPLC–DAD 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 ^{3D}Chemstation (Revision A.02.05).

2.3. Chromatographic conditions

The separation was performed by the following multi-step gradient developed by using CARTAGO-S (Computer Assisted Retention Time Prediction And Gradient Optimization-Spreadsheet) [18–20]: 0–2.5 min: 2.0–2.5%; 2.5–2.8 min: 2.5–17.5%; 2.8–3.5 min: 17.5–2.5%; 5.0–6.0 min: 2.5–7.5%; 6.0–7.5 min: 7.5–14.5%; 7.5–11.0 min; 14.5–25% B in A+B. A: water, containing 8.5 g of *o*-phosphoric acid (85%) and 0.39 g of hexylamine per 1000 g; B: acetonitrile–water (695 g: 280 g), containing 8.5 g of *o*-phosphoric acid (85%) and 1.1 g of hexylamine per 1000 g. The reconditioning time was 15 min. A 125×2-mm I.D. column with a 20×2-mm I.D. guard column, packed with 3-μm Spherisorb C_{18} ODS-2 (Stagroma, Wallisellen, Switzerland) was used. The flow-rate was 0.2 ml/min and the temperature was set at 40°C. Detection and quantitation were performed at 210 nm. Online DAD spectra were recorded from 192 to 350 nm and used for peak purity check and peak assignment by library match.

2.4. Calibration

The quantitative determination 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 by measuring aqueous solutions of pure standards at known concentrations. Each calibration graph was calculated by linear regression analysis from triplicate determinations of four concentration levels in the range of 25 to 5000 ng/ml. The calibration samples were stored at −18°C.

2.5. Extraction procedure

The extraction of plasma samples was performed on a Nucleosil C_{18} ODS-2 solid-phase extraction column (SPE; Macherey-Nagel, Oensingen, Switzerland), conditioned with 10 ml of methanol, followed by 5 ml of 40% acetonitrile in 0.01 *M* phosphate buffer (pH 2.1) and 10 ml of bidistilled water. To 1.5 ml of the plasma samples, 4.5 ml of a 0.5 *M* carbonate buffer (pH 9.3) and 150 μl of a 0.1% aqueous solution of I.S. were added and vortexed for 10 s. 4.1 ml of this solution were 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 40% acetonitrile in 0.01 *M* phosphate buffer (pH 2.1). For the elution of the opiates and opiate metabolites two portions of 0.6 ml of 40% acetonitrile in 0.01 *M* phosphate buffer (pH 2.1) were applied on the SPE column.

The total volume of 1.2 ml of the eluate was evaporated to dryness, reconstituted in 100 μl of bidistilled water and 2 μl were injected into the HPLC.

2.6. Precision

A blank plasma sample was spiked with 50, 500 and 5000 ng/ml of the compounds of interest, divided in portions and frozen at −18°C. Every week eight control samples were treated as described above and each sample was analyzed in duplicate.

The calibration and the efficiency of the chromatographic system were checked every week by analyzing the stored calibration samples.

2.7. Sample collection

Blood samples (10 ml) from a patient (subject A) participating in a pilot study of the Heroin Maintenance Program of KODA-1 in Bern were collected by a peripheral vein catheter 10 min before (blank) and 1, 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60 and 120 min after the intravenous self-injection of 200 mg DAM. After centrifugation (2500 *g*, 10 min) the plasma samples were stored at −40°C. Blood samples of the dialysis patient (subject B) at the University Hospital of Bern were collected 1 h before and 1, 2, 8, 22, 35, 45, 52, 66, 76, 92, 95, 99, 118, 145, 168 h after the beginning of the self-administration of a total dosage of 110 mg *M*. After centrifugation, the plasma samples were stored at −40°C.

3. Results and discussion

3.1. HPLC system

C_{18} reversed-phase materials with *o*-phosphoric acid, acetonitrile and hexylamine as mobile phase have shown good selectivity and reproducibility for the separation and quantitation of a wide range of basic compounds and have been used successfully in our laboratory for the determination of psychoactive substances [21–24]. Hexylamine serves as a masking agent for free silanol groups resulting in improved peak shape and smaller k' values.

CARTAGO-S, a spreadsheet-based computer program, can be used for chromatogram simulation and gradient optimization [18–20]. It exploits the fact that the logarithm of the capacity factor k' varies approximately linearly with the concentration of the organic modifier. The parameters of this linear relationship are determined for each compound by at least two isocratic runs, and are then used to predict the retention behavior of these compounds. Using the CARTAGO-S software, time-consuming experimental evaluation can be minimized. The resulting multi-step gradient allows the differentiation of opiates within a wide range of polarity in less than 20 min. Fig. 1 shows the chromatograms of a standard mixture predicted by CARTAGO-S vs. that obtained under real conditions by HPLC.

Peak assignment was achieved by standards and DAD-UV spectra. The low UV cut-off of the mobile phase allows the detection at 210 nm where all opiates analyzed exhibit a UV maximum. Online UV-spectra of M3G, M6G, 6AM and DAM are shown in Fig. 2B. Typical chromatograms of a blank plasma extract, a plasma extract obtained 2 min after injection of 200 mg DAM (subject A) and a plasma extract of a comatose patient (subject B) 45 h after starting with self-administration of M are shown in Fig. 2A–C. The endogenous compound at approximately 15 min is always more or less present but

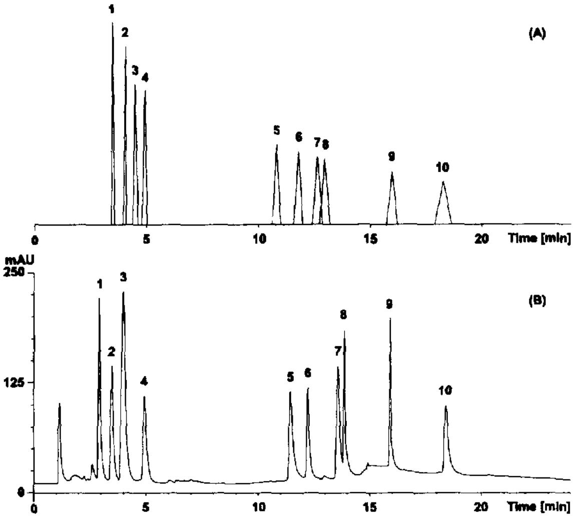

Fig. 1. Predicted (A) and real (B) chromatogram of a standard mixture. (1) NM, (2) M3G, (3) M, (4) M6G, (5) C, (6) C6G, (7) 3AM, (8) 6AM, (9) I.S., (10) DAM.

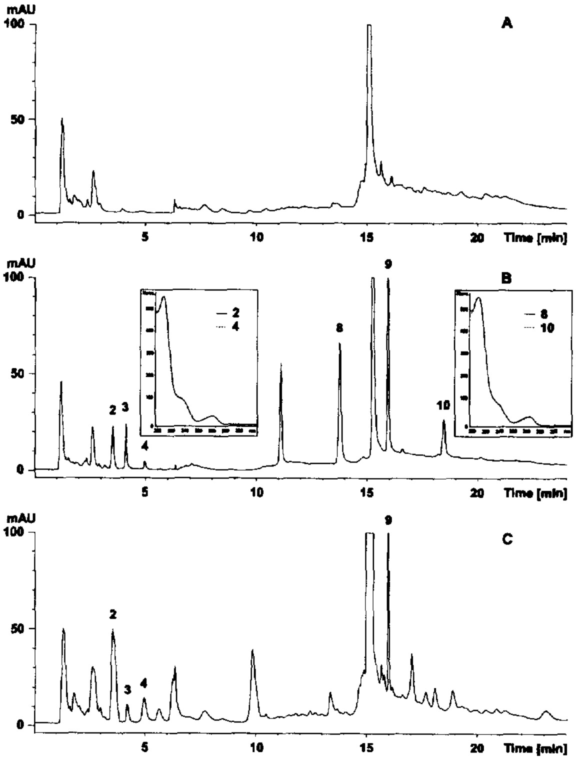

Fig. 2. Chromatograms of plasma extracts. A: blank, B: 2 min after injection of 200 mg of DAM (subject A), C: 45 h after starting with the self-administration of M (subject B).

does not interfere with the I.S. As EM was a commercially available standard and the subjects participating in the "Heroin Maintenance Programs" were by contract strictly not allowed to consume other opiates, the latter was chosen as I.S. With a retention time of 6.6 min, the tested DHM proved to be a good alternative for EM as internal standard.

3.2. Calibration

Calibration curves were linear in the range of 25 to 5000 ng/ml, and the regression equation showed correlation coefficients of >0.998. The calibration proved to be stable over several months. Due to its instability, 3AM was not calibrated and could not be detected in all analyzed plasma samples.

3.3. Precision and recovery

The extraction efficiency is shown by the good to excellent recovery which varied from 81 to 101% (see Table 1). The intra- and inter-day precision was between 1.0 and 5.0% (n=8). The reproducibility data are summarized in Table 1. The limit of quantitation (LOQ) was 25 ng/ml for each compound with a signal-to-noise ratio >7, a peak purity >99% and a library match for the UV spectra

Table 1
Recovery and precision of the method

Compound	Recovery (n=8) (%)	Precision (n=8) (C.V., %)					
		Intra-day			Inter-day		
		50 ng/ml	500 ng/ml	5000 ng/ml	50 ng/ml	500 ng/ml	5000 ng/ml
Normorphine (NM)	88	2.0	3.3	4.2	2.2	4.2	4.6
Morphine-3-*O*-glucuronide (M3G)	91	2.5	3.4	3.9	3.5	4.1	5.0
Morphine (M)	98	1.6	3.2	4.0	2.3	4.3	4.3
Morphine-6-*O*-glucuronide (M6G)	90	2.2	4.6	3.7	3.2	4.9	4.7
Codeine (C)	91	2.2	3.2	3.4	2.9	3.2	4.4
Codeine-6-*O*-glucuronide (C6G)	100	4.2	3.2	3.5	4.8	4.2	4.1
3-Acetylmorphine (3AM)	–	–	–	–	–	–	–
6-Acetylmorphine (6AM)	94	2.5	3.4	3.5	3.4	3.5	4.4
Ethylmorphine (I.S.)	82	1.2	1.3	1.8	1.6	1.3	2.1
Diacetylmorphine (DAM)	88	3.1	3.5	3.7	3.3	4.2	4.8

>99.8%. The LOQ was by far low enough to establish plasma profiles after the administration of high doses of DAM and M.

3.4. Analysis of plasma samples

1 min after injection of 200 mg DAM, peak concentrations of 1490 ng/ml DAM and 3410 ng/ml 6AM were measured in plasma samples of subject A, receiving DAM. Due to the short plasma half-life of DAM ($t_{1/2}$: 9 min [25]) and 6AM ($t_{1/2}$: 38 min [25]) the concentration of these compounds decreased rapidly within 2 min to 814 ng/ml and 1887 ng/ml, respectively. M reached its peak concentration of 166 ng/ml after 6 min. The highest levels of M3G, the dominating and most persisting metabolite of DAM, and M6G were 3850 ng/ml and 399 ng/ml, respectively, appearing 15 min after intravenous administration. The plasma profiles are shown in Fig. 3.

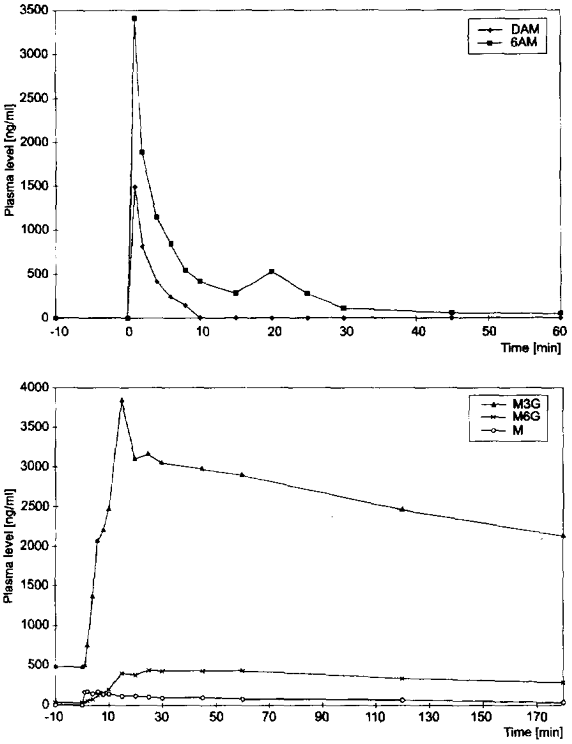

Fig. 3. Plasma profiles after injection of 200 mg of DAM (subject A).

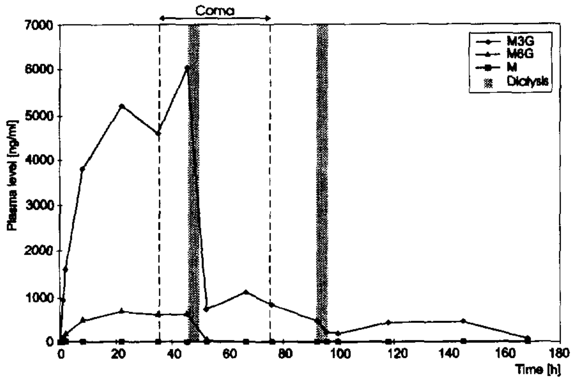

Fig. 4. Plasma profiles after self-administration of M (subject B).

Fig. 4 shows the plasma curves of the self-intoxication case with M. Peak concentrations of 6050 and 630 ng/ml M3G and M6G, respectively, were found in plasma samples of the dialysis patient (subject B) 45 h after the beginning of self-administration of a total dosage of 110 mg M. With 92.3 ng/ml, the peak plasma concentration of M appeared 1 h after the start of self-administration, whereas after 45 h no M could be detected. The dramatic decrease of the M3G and M6G plasma levels from 6050 to 250 ng/ml and from 625 to 21 ng/ml, respectively, was the result of the dialysis treatment. The patient started to recover slowly after 76 h.

4. Conclusions

The described HPLC–DAD method represents a rapid and sensitive method for the determination of DAM, M, C and their major and minor metabolites in human plasma. It cannot only be used for pharmacokinetic studies but also for monitoring clinical intoxication cases and drug abuse. A LC–MS method with atmospheric pressure ionization is currently under development. The improved sensitivity and specificity of this powerful technique should allow therapeutic and forensic applications, too.

Acknowledgments

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