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Quantitative analysis of morphine in human plasma by gas chromatography–negative ion chemical ionization mass spectrometry

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Abstract

A sensitive and specific method for the quantitative determination of morphine in human plasma is presented. Morphine was extracted from plasma by solvent extraction with ethyl acetate and derivatized to its heptafluorobutyrate derivative. The derivatives were measured by gas chromatography–negative ion chemical ionization mass spectrometry without any further purification. Using this detection mode, a diagnostic useful fragment ion at m/z 637 is obtained at high relative abundance. Deuterated morphine was used as an internal standard. Calibration graphs were linear within a range of 0.78 ng/ml and 50 ng/ml. Inter-assay precision was 2.3% (2.85 ng/ml) and 1.4% (14.25 ng/ml), intra-assay variability was found to be 1.5% (3.71 ng/ml) and 0.5% (14.54 ng/ml). Accuracy showed deviations of −9.3% (2.85 ng/ml) and −4.2% (14.25 ng/ml). The method is rugged and robust and has been applied to the batch analysis of morphine during pharmacokinetic profiling of the drug.

Keywords: Morphine

1. Introduction

Morphine is a potent opioid analgesic which is used for short-term treatment of postsurgical and traumatic pain as well as for long-term treatment of severe pain in cancer patients [1]. Besides these clinical applications, morphine is a common drug of abuse. In humans it is mainly metabolized to the 3- and 6-glucuronide [2,3]. Several different methods for the determination of morphine in plasma have been described. Immunological methods [4] are prone to cross-reactivity towards morphine metabolites and are thus of limited use [5]. Liquid chromatography (LC) with electrochemical detection has also been used, but is rather time consuming [6,7]. For the simultaneous measurement of morphine and its glucuronide metabolites, LC–MS methods have recently been described [8–10]. The required specificity and sensitivity is met by gas chromatography (GC) in combination with mass spectrometry (MS) [11–13] or electron capture detection [14]. Besides the use of electron ionization (EI), positive-ion

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chemical ionization methods have also been employed for the analysis of morphine [15]. Negative-ion chemical ionization was used for the determination of morphine in plasma of children after hydrolysis of their glucuronides [16] and as a primary ionization method for subsequent analysis by tandem mass spectrometry [13].

For pharmacokinetic applications robustness and short analysis time is a major concern. The expected plasma concentrations during these studies usually range from 0.8 to 20 ng/ml, and involve the processing of a large a number of samples. It was therefore the aim of this study to elaborate a method for the determination of morphine in human plasma that meets the requirements of sensitivity, specificity, speed and ruggedness for pharmacokinetic applications.

2. Experimental

2.1. Chemical and reagents

Morphine·HCl and morphine-(*N*-methyl-d_3) (d_3-morphine) were purchased from Lipomed (Arlesheim, Switzerland). Heptafluorobutyric anhydride was from Sigma (Vienna, Austria). All other solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

2.2. Gas chromatography–mass spectrometry

A Finnigan TRACE GC coupled to a Finnigan Voyager quadrupole MS (ThermoQuest, Vienna) was used. The GC was fitted with a DB-5MS fused-silica capillary column (15 m×0.25 mm I.D., 0.25-μm film thickness, ThermoQuest). The injector was operated in the splitless mode at 280°C. Helium was used as a carrier gas at a constant flow-rate of 1.5 ml/min. Initial column temperature was 120°C for 1 min, followed by an increase of 40°C/min to 310°C and an isothermal hold of 2 min. The mass spectrometer transfer line was kept at 315°C. Negative-ion chemical ionization (NICI) was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 0.250 A. During single-ion recording, *m/z* 637 and *m/z* 640 were recorded for target and internal standard, respectively, with a dwell time of 50 ms.

2.3. Plasma sample preparation

Fifty μl of the methanolic solution of the internal standard, containing 17.4 pmol d_3-morphine·HCl are pipetted into a 5-ml polypropylene tube and 0.5 ml of plasma added. After short vigorous shaking, 1 ml of buffer (10 m*M* ammonium carbonate, pH 9.3) is added, the samples are equilibrated for 10 min and 2.5 ml of ethyl acetate are added. The tubes are stoppered and shaken on a reciprocal shaker for 15 min. After centrifugation at 3500 rpm for 10 min, the (upper) organic layer is transferred to a clean conical-shaped polypropylene tube and dried under nitrogen. The dry residue is treated with 100 μl of reagent solution (heptafluorobutyric anhydride (HFBA)–acetonitrile–ethyl acetate, 1:2:0.5, v/v/v) for 15 min at room temperature. After evaporation of the reagents under nitrogen, the residue is dissolved in 100 μl of ethyl acetate and transferred to autosampler vials. The vials are closed with crimp-top caps and stored at −20°C until analysis. Derivative stability was checked by analyzing samples immediately and after storage at room temperature and −20°C.

2.4. Recovery of morphine after solvent extraction

To estimate analyte recovery during solvent extraction, morphine-free blank plasma was spiked with 14.25 ng/ml of morphine and extraction carried out as described in Section 2.3. After extraction 5.01 ng of d_3-morphine·HCl internal standard were added to the ethyl acetate phase and the sample derivatized as described. To estimate the maximal value (100%), 14.25 ng/ml of morphine were mixed with 5.01 ng d_3-morphine·HCl internal standard and derivatized directly. Five-fold determinations were carried out. We have also investigated ethyl acetate extraction at pH 9.3, neutral pH and pH 3.0.

2.5. Analytical method validation

Calibration graphs were established in the range of 0.78 to 50 ng/ml of plasma. For this purpose, blank plasma was spiked with the appropriate amounts of

morphine by adding 50 μl of the corresponding methanolic solution. Standard solutions of morphine were prepared by serial dilution in methanol to yield concentrations of 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 ng/50 μl. Standard solutions were stored at −20°C. Blank plasma was checked for possible morphine content before use.

The instrumental detection limit was estimated by derivatizing 28.5 ng of pure morphine sample and injecting diluted aliquots into the GC–MS system.

Inter-assay precision was determined at 2.85 ng/ml plasma and 14.25 ng/ml plasma by carrying identical samples throughout the analytical sequence. Spiked samples were prepared from blank plasma. Ten-fold determinations were carried out.

Intra-assay precision was determined at 3.71 ng/ml plasma and 14.54 ng/ml plasma by multiple GC−MS analysis of one prepared sample. Spiked samples were prepared from blank plasma. Six-fold determinations were carried out.

Accuracy of the methods was also tested at the above-mentioned concentrations after ten-fold determinations. Thus, the data from inter-assay precision measurements were used to calculate the deviation of the values measured from the actual spiked values.

Specificity was tested by analyzing ten different blank plasma samples and samples spiked with various drugs.

To measure freeze–thaw stability, plasma samples were analyzed immediately after spiking with the indicated amounts of morphine and after three freeze–thaw (F–T) cycles.

Quality control samples were prepared at concentrations 2.85, 14.25 and 42.75 ng/ml of plasma by spiking blank plasma pools with the appropriate amounts of morphine. These samples were analyzed in duplicate at different days to calculate between-day precision and stability data.

3. Results and discussion

3.1. Sample preparation

The extraction procedure described here offers a rapid way to isolate morphine from the plasma matrix. Thus, we have achieved extraction and derivatization of batches with 112 samples routinely within 5 h, with two technicians working on it.

The amphoteric nature of morphine requires careful adjustment of pH. Extraction at alkaline pH, as frequently used for simultaneous extraction of morphine and its metabolites, produced the cleanest extract in terms of background interference in the chromatograms. Analyte recovery was rather low at 53.0% (Table 1), but the high sensitivity of the assay together with the use of deuterated morphine as an internal standard rendered this problem as minimal. Although extraction proceeded not quantitatively, the procedure was highly reproducible (C.V. 3.3%) since loss during extraction affects target and internal standard as well. Thus, in the light of the analytical requirements and performance, this rapid extraction procedure was favoured. Derivatization occurred quantitatively within 15 min using the reagent/solvent system described. The derivative was stable for at least three weeks at −20°C and three days at room temperature. Samples were stored at −20°C to assure unattended possibility of sample re-analysis over a whole time range of batch sample processing.

3.2. Mass spectrometry

The NICI spectra of the heptafluorobutyrate derivative of morphine and d_3-morphine are shown in Fig. 1A and B, respectively. The mass spectrum of the morphine derivative shows prominent high-mass fragment ions at m/z 657 and m/z 637, resulting from loss of HF twice from the molecular ion. From these ions, m/z 637 was used as a quantitation mass due to its low background under NICI detection. The corresponding ion of the internal standard was at m/z

Table 1
Inter- and intra-assay precision and accuracy of morphine determination in human plasma[a]

	Inter-assay		Intra-assay	
	(ng/ml)	(ng/ml)	(ng/ml)	(ng/ml)
Spiked amount	2.85	14.25	3.71	14.54
Mean	2.59	13.66	3.73	14.58
SD	0.059	0.198	0.057	0.068
n	10	10	6	6
C.V.	2.3%	1.4%	1.5%	0.5%
Recovery	90.7%		95.8%	

[a] Accuracy was calculated from inter-assay data.

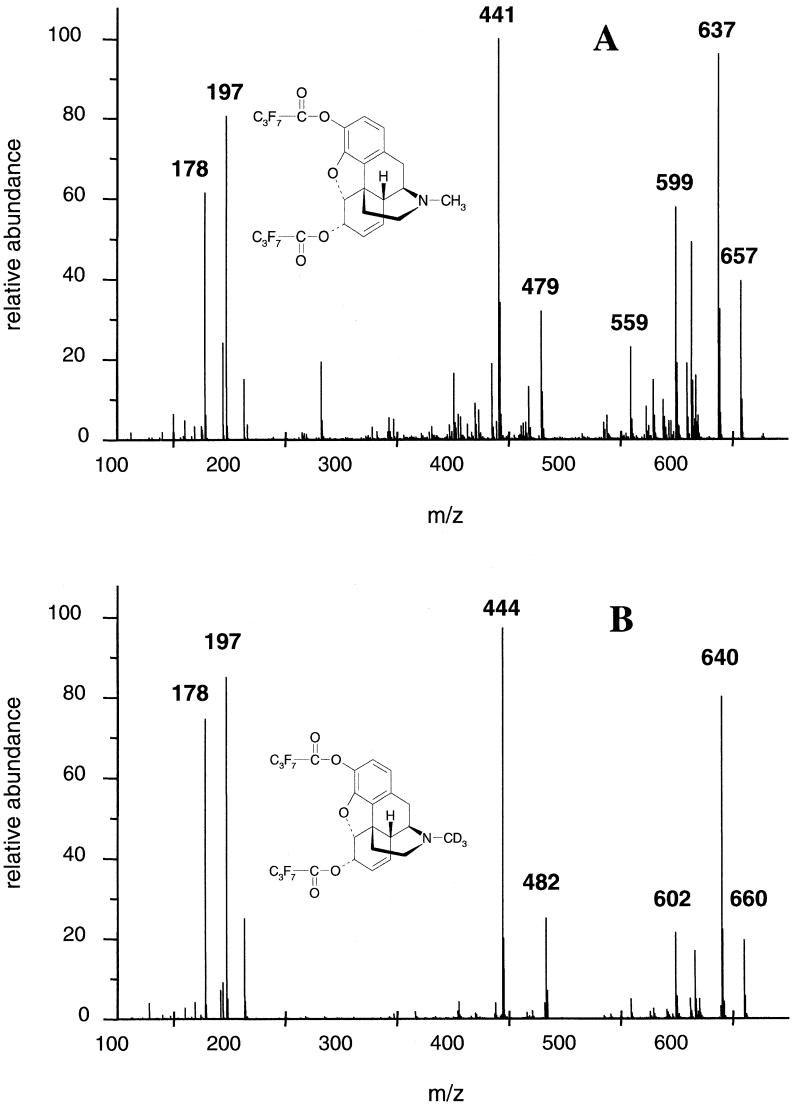

Fig. 1. NICI mass spectrum of (A) morphine and (B) d_3-morphine after conversion to the heptafluorobutyrate derivative.

640. The use of triple deuterated internal standard, however, may be a potential source of systematic error. Analyzing plasma spiked only with various amounts of unlabeled morphine, we have found a signal at m/z 640 corresponding well with the theoretically expected isotopic pattern of the fragment ion used for quantification ($[C_{25}H_{15}F_{12}NO_5]^{\cdot -}$, M+3=0.5%). Thus, using the highest concentration of morphine in the calibration range (50 ng/ml) and 5.01 ng of d_3-morphine internal standard, the area ratio of sample/standard is reduced from 9.98 to 9.51. This would cause an ideal linear regression correlation coefficient of $r=1.0000$ to be reduced to $r=0.99992$. This inherent systematic error can be minimized by using higher amounts of internal standard and/or using suitable non-linear regression analysis. The problem has also been recognized by other groups [9]. In our experience, however, this error can be minimized and is by far overrided by the benefits of a deuterated internal standard.

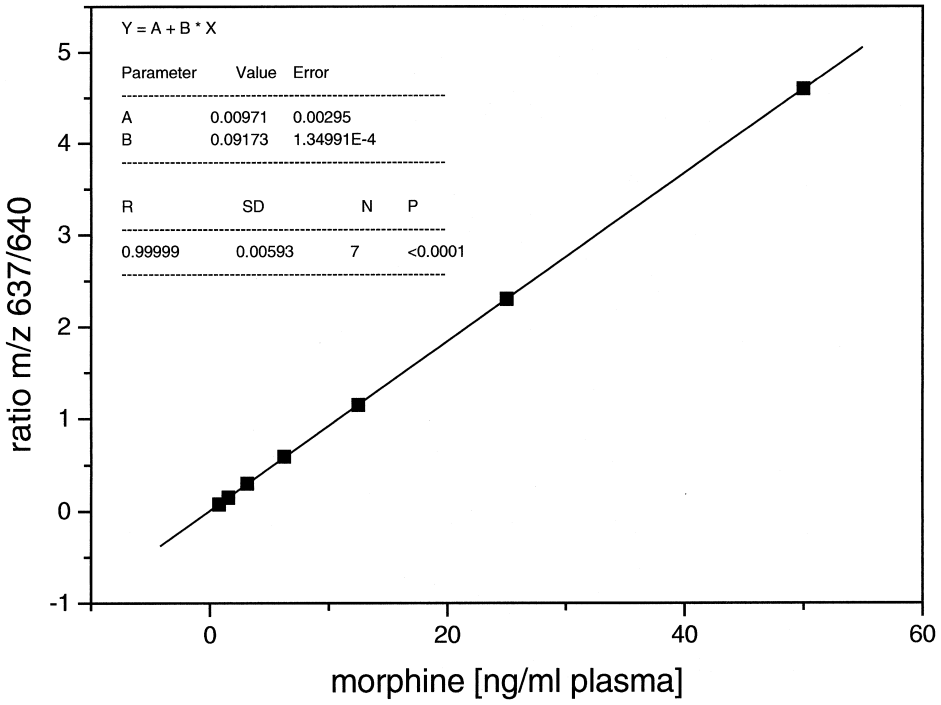

Fig. 2. Calibration graph and statistics for the determination of morphine in human plasma.

3.3. Analytical method validation

The calibration graphs established were linear within the tested range of 0.78 ng/ml plasma to 50 ng/ml plasma, using 5.01 ng of d_3-morphine·HCl internal standard.. A typical calibration curve with the statistical data is shown in Fig. 2. The minimum detectable injected quantity of the mass spectrometer was found to be 5 pg at a signal-to-noise ratio of 4:1. The lower limit of detection for the complete procedure was 0.29 ng/ml plasma, estimated by analyzing spiked plasma samples. For routine measurements, the limit of quantitation (LOQ) was set to 0.78 ng/ml plasma.

The coefficients of inter- and intra-assay variation and the analyte recovery of the spiked samples are presented in Table 1. It can be seen from these data, that the method provides a highly precise assay for morphine in human plasma. This can be attributed to the inherited sensitivity of NICI detection in combination with a stable isotope-labeled internal standard. Mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and assays based on this technique can be regarded as reference procedures to validate other analytical methods.

No decomposition of standard and stock solutions was measurable after two months of storage. Investigations on freeze–thaw stability of plasma samples at three different concentrations did not

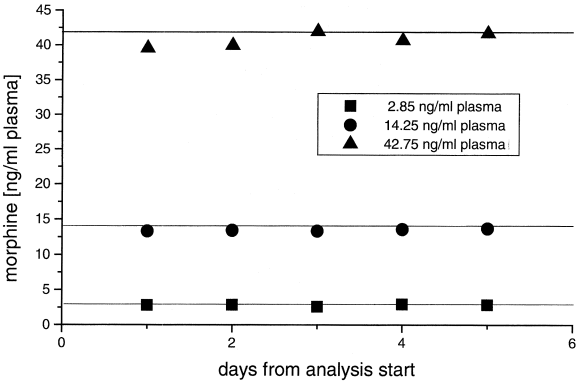

Fig. 3. Stability of quality control samples at 2.85, 14.25 and 42.75 ng/ml plasma. Samples from prepared spiked plasma pools were analyzed on five consecutive days in duplicate.

Table 2
Freeze–thaw stability of plasma samples containing morphine[a]

Amount found before three F–T cycles (ng/ml)	Amount found after three F–T cycles (ng/ml)	% found after three F–T cycles
50.62	50.67	100.1
17.78	17.03	95.8
3.47	3.57	102.8

[a] Plasma samples were analyzed immediately after spiking with the indicated amounts of morphine and after three freeze–thaw (F–T) cycles.

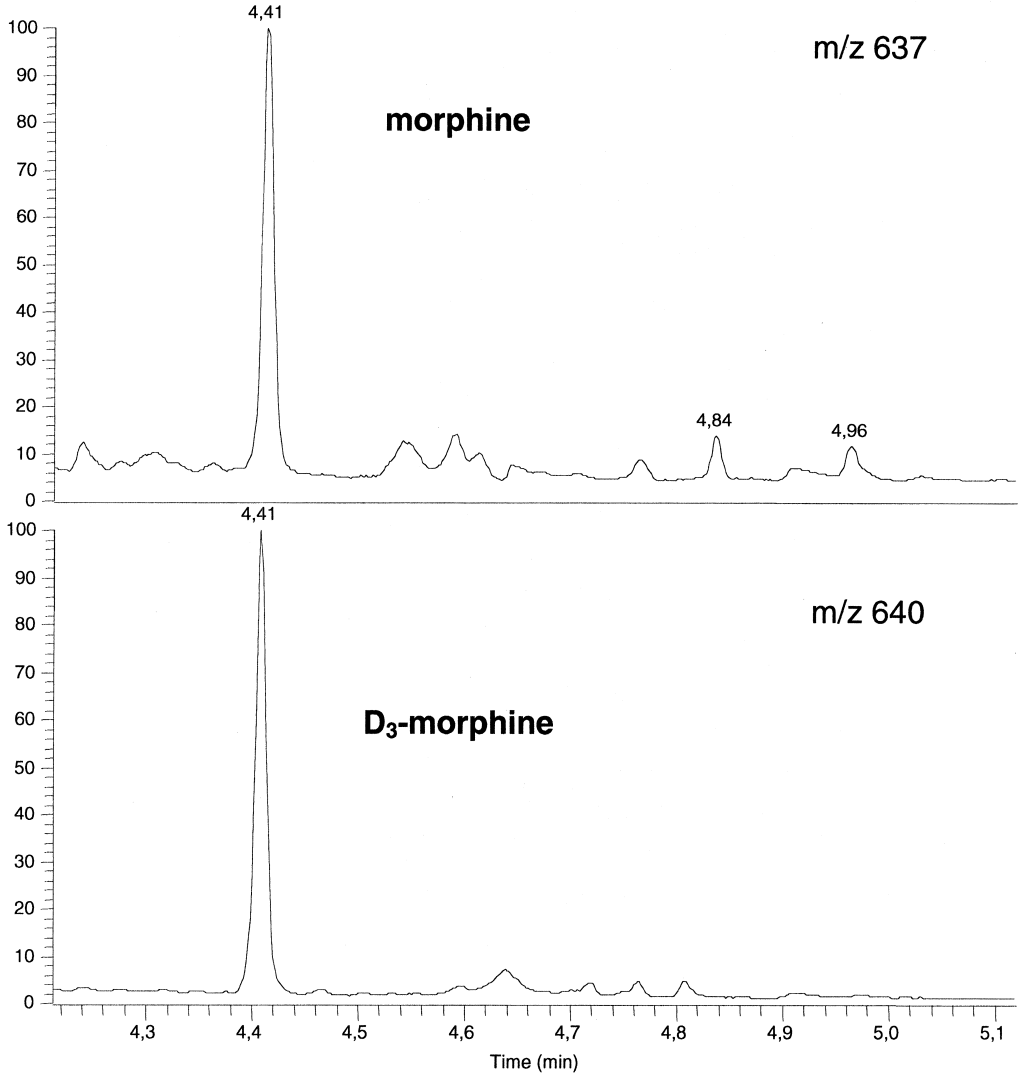

Fig. 4. SIR mass chromatogram obtained after analysis of a plasma sample from a human volunteer receiving morphine orally. The corresponding plasma level is 3.42 ng/ml.

indicate any sample degradation after three freeze–thaw cycles. The results are shown in Table 2. Ten different blank matrices were checked for interferences. In none of the samples there was background contribution above 25% LOQ. Samples containing lisinopril (50 ng/ml), enalaprilate (80 ng/ml), diclofenac (50 ng/ml), ketoprofen (30 ng/ml), captopril (50 ng/ml), terbutaline (10 ng/ml), nitrendipine (30 ng/ml) or methylphenidate (10 ng/ml) did not show any interference with the measurement of morphine.

Between-day precision was estimated to be 4.71%, 1.19%, and 2.63% at 2.85, 14.25 and 42.75 ng/ml plasma, respectively. Stability during analytical batch processing was assessed at five consecutive days. The results are shown in Fig. 3.

We have applied this method to the analysis of morphine in human plasma in the course of a pharmacokinetic study. A typical mass chromatogram obtained after analysis of a plasma sample after oral administration of morphine is given in Fig. 4. The assay proved to be useful in the batch analysis of more than 300 plasma samples. The low amounts of sample and solvents needed due to the sensitivity of the NICI detection allow rapid sample preparation and assure a high throughput in analysis. Under the conditions described, the morphine derivative elutes after 4.4 min from the GC. The total analysis cycle including cooling of the GC and equilibration time is 12 min per sample.

The analysis of morphine from human plasma is of major interest in pharmaceutical research and forensic and toxicological monitoring of the drug. It is however noteworthy, that the 6-β-D-glucuronide of morphine possesses analgesic properties even higher than the parent drug, thus necessitating the differential analysis of both, morphine and its metabolites [17,18]. Nevertheless, in pharmaceutical sciences the rapid determination of morphine pharmacokinetics can provide preliminary data on the bioavailability of new formulations.

Acknowledgements

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