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Note

Determination of morphine in plasma by gas chromatography using a macrobore column and thermoionic detection after Extrelut column extraction: application to follow-up morphine treatment in cancer patients

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Morphine is currently used to treat severe pain in cancer patients. Tablets with sustained release of morphine are commercially available and control of morphine levels is needed in order to compromise between pain relief and retention of consciousness.

A variety of techniques for the determination of morphine in biological fluids have been reported. Thin-layer chromatography is rapid and inexpensive but its sensitivity is only about 0.50 mg/l [1], although the use of dabsylation greatly improves the sensitivity [2]. Radioimmunoassay procedures are simple, allowing a large number of assays in series, and are very sensitive (picogram amounts can be detected), but often lack specificity owing to cross-reaction between morphine and its metabolites [3,4]. Recently, several high-performance liquid chromatographic methods have been proposed [5], which can separate morphine from its metabolites (morphine-3- and -6-glucuronide) [6]. Gas chromatography (GC) with electron-capture detection is both sensitive and specific [7,8], but contamination of the detector frequently occurs. Gas chromatography–mass spectrometry (GC–MS) is as sensitive as radioimmunoassay and has an unsurpassed specificity [9,10], although it requires expensive apparatus.

This paper describes a GC method for the determination of free and total mor-

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phine, using a macrobore column, a thermionic detector, extraction with an Extrelut column and trifluoroacetate derivatives. This method is sufficiently sensitive for the determination of morphine concentrations in plasma from cancer patients treated orally with Moscontin (Sarget, Merignac, France) (tablets of morphine sulphate with a sustained release of morphine). GC–MS was used to check that the peak identified as morphine in the plasma did not contain interfering compounds.

EXPERIMENTAL

Reagents and materials

Ethyl acetate, sodium acetate, acetic acid (all of analytical-reagent grade) and columns packed with Extrelut silica (Extrelut No. 3) were purchased from Merck (Darmstadt, F.R.G.). Nalorphine, used as an internal standard for the quantitative evaluation, was supplied by Clin Midy (Paris, France). Enzyme solution from *Helix pomatia* (100 000 Fishman U/ml β-glucuronidase and 1 000 000 Roy U/ml sulphatase) was obtained from the Institut Pasteur (Paris, France). Pethidine was obtained from Specia (Paris, France), dextromorphan, methadone and codeine from Sigma (St. Louis, MO, U.S.A.), heroin from Coopérative Pharmaceutique Française (Melun, France) and hydromorphone and oxycodone from Francopia (Paris, France).

Gas chromatography

An AIGC 121 DFL gas chromatograph (Delsi, Suresne, France) equipped with a rubidium ball thermionic detector (a nitrogen–phosphorus-selective detector) was used. The GC separation was achieved using a 10 m$\times$0.53 mm I.D. column of SE 54 fused-silica (Spiral, Dijon, France). The injector and detector temperatures were 250 and 300°C, respectively. Chromatograms were developed with temperature programming from 120 to 250°C at 10°C/min. The flow-rate of the carrier gas (nitrogen) was 25 ml/min.

Gas chromatography–mass spectrometry

The assays were run on an LKB (Bromma, Sweden) Model 2091 apparatus equipped with an SE-30 column (250$\times$0.034 cm I.D.) (Spiral). Helium was used as the carrier gas. The injector, separator and source temperatures were 245, 245 and 260°C, respectively. Mass fragmentation was obtained in the electron-impact mode (ionization energy 22 eV). The temperature was programmed from 160 to 250°C at 5°C/min. Characteristic fragments from morphine were *m/z* 477 (M^+) and 364 ($M^+ - CF_3COO$).

Sample preparation

Samples of 5 ml of blood were taken in heparinized tubes and free morphine was assayed in 1 ml of plasma. Total morphine (conjugated+free) was determined in 1 ml of hydrolysed plasma.

Hydrolysis. A 1-ml volume of plasma was spiked with 1.25 μg of nalorphine (internal standard) before hydrolysis. The pH was adjusted to 5.2 with 10% acetic

acid (1.7 *M*) and 0.5 ml of 2 *M* acetate buffer (pH 5.2) was added. The sample was hydrolysed with 25 μl of crude solution from *Helix pomatia* at 37°C for 24 h.

Extraction procedure and derivatization. The pH of the sample obtained was adjusted to 10 with 12 *M* sodium hydroxide solution. Each sample was loaded onto an Extrelut column and after 15 min elution was carried out twice with 10 ml of ethyl acetate. The organic solvent was evaporated to dryness under a stream of nitrogen at 37°C and the dry residue was derivatized with 100 μl of trifluoroacetic anhydride (TFAA) in ethyl acetate (1:1) by heating at 60°C for 20 min. After cooling, the organic phase was evaporated to dryness under a stream of nitrogen at 37°C. The dry residue was dissolved in 50 μl of ethyl acetate and 1 μl was injected directly on-column with a Hamilton (Bonaduz, Switzerland) syringe. For the assay of free morphine, the dry residue was dissolved in 25 μl of ethyl acetate and 2 μl were injected.

Subjects

Pharmacokinetic studies were carried out on five cancer patients (29–61 years old). Two subjects received orally 3×230 mg per day of Moscontin and three others received 2×90, 2×70 and 2×30 mg per day of Moscontin. The pharmacokinetic studies were performed when the patients were in a steady state. Blood was collected just before an oral dose and 2, 4 and 8 h after drug absorption.

Renal function was investigated on the basis of serum urea and creatinine (normal values are <8.5 mmol/l and <114 μmol/l, respectively). Liver function was evaluated as bilirubin, alanine aminotransferase and alkaline phosphatase (normal values are <17 μmol/l, <25 I.U./l and 70–210 I.U./l, respectively).

RESULTS

Chromatographic data

Representative chromatograms of morphine in aqueous solution and in plasma are shown in Fig. 1. No interfering endogenous compounds are apparent in the plasma before or after enzymatic hydrolysis (Fig. 2).

Fig. 3 shows a chromatogram of plasma from a patient before (free morphine) and after hydrolysis (total morphine). The morphine and nalorphine peaks are well resolved. The retention times of morphine and nalorphine are 10.54 ± 0.06 min ($n=10$) and 11.78 ± 0.08 min ($n=10$), respectively. The relative retention times (morphine-to-nalorphine retention time ratio) are similar from an aqueous standard solution [0.880 ± 0.005 ($n=10$)] and morphine-spiked plasma [0.880 ± 0.006 ($n=10$)]. The response coefficients of morphine (morphine-to-nalorphine peak-area ratio) are also similar for an aqueous standard solution and plasma [1 ± 0.005 ($n=30$) and 1.001 ± 0.006 ($n=30$), respectively].

Calibration graphs were prepared by adding 0.1–20 μg of morphine and a constant amount of nalorphine (1.25 μg) to 1 ml of water or 1 ml of drug-free hydrolysed or non-hydrolysed plasma. The aqueous standard solutions and plasmas were extracted and derivatized as described under Experimental. The concentration–response relationship is linear between 2 and 200 ng/μl injected. Linear regression analysis on calibration graphs obtained with distilled water and plasma

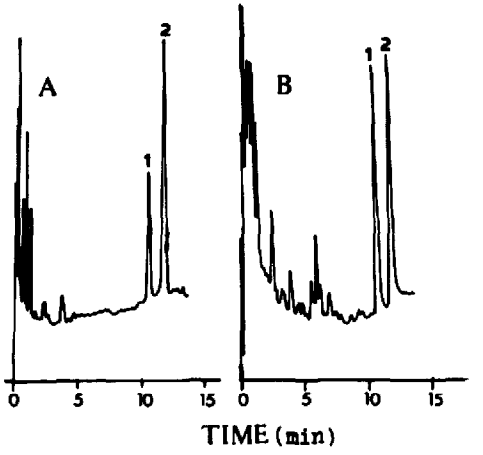

Fig. 1. Gas chromatograms of (A) aqueous standard solution of morphine (0.67 μg/ml) and internal standard nalorphine (1.25 μg/ml) and (B) plasma spiked with morphine (1.25 μg/ml) and nalorphine (1.25 μg/ml). Peaks: 1 = morphine; 2 = nalorphine.

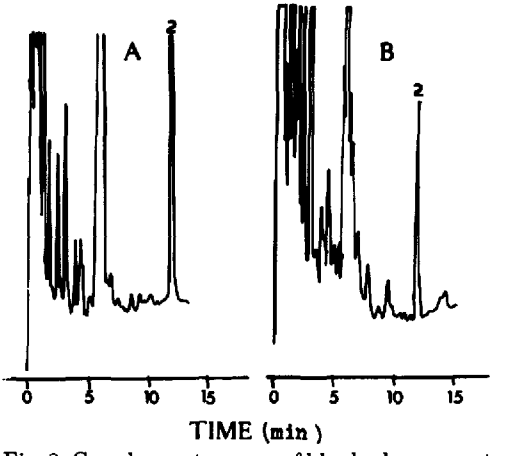

Fig. 2. Gas chromatograms of blank plasma containing nalorphine (1.25 μg/ml) (A) before hydrolysis and (B) after hydrolysis. Peak 2 = nalorphine.

indicated that the correlation coefficients were 0.994 and 0.987, respectively, and the intercepts were near the origin (-0.03 and $+0.02$, respectively). Routinely, we used an aqueous standard solution to establish the calibration graphs. This concentration range allowed the detection of the amounts of morphine currently encountered in plasma during therapeutic monitoring.

The detection limit was 2 ng of pure sample injected with a signal-to-noise ratio of 3:1.

The recovery was determined by comparing the peak area of morphine added to a plasma sample extracted and not extracted with that of nalorphine (not extracted). The recoveries were $67 \pm 9\%$ ($n=5$), $76 \pm 10\%$ ($n=6$) and $87 \pm 2\%$ ($n=5$) at levels of 0.5, 1.25 and 2.5 μg/ml, respectively. To compensate for losses during extraction, internal standard was added to the sample before hydrolysis and extraction.

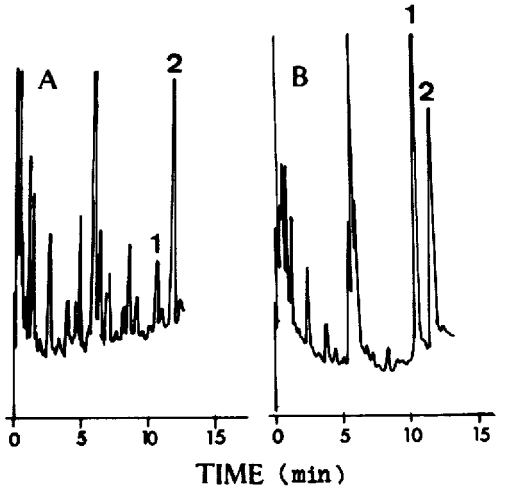

Fig. 3. Gas chromatograms of a patient plasma treated with Moscontin. (A) Free morphine, 0.60 μg/ml; (B) total morphine, 7.2 μg/ml. Peaks: 1 = morphine; 2 = nalorphine.

TABLE I

ORDER OF RETENTION AND RETENTION TIME RELATIVE TO NALORPHINE OF VARIOUS DRUGS

Compound	Retention time (min)	Relative retention time
Pethidine	4.38	0.372
Methadone	9.02	0.767
Dextromethorphan	9.09	0.772
Morphine	10.54	0.880
Codeine	10.74	0.910
Nalorphine	11.78	1.000
Heroin	13.72	1.163
Hydromorphone[a]	-	-
Oxycodone[a]	-	-

[a]Hydromorphone and oxycodone were not extracted with this method.

The within-day precision of the assay was established by replicate analyses of plasma samples spiked with 0.5 and 1 μg/ml morphine. The coefficients of variation were 3.37% ($n=10$) and 3.25% ($n=10$), respectively. The day-to-day precision was evaluated on ten different days. The coefficient of variation was 4.75% for 0.5 μg/ml and 5% for 1 μg/ml.

Interferences from pethidine, methadone, codeine, dextromethorphan, heroin, hydromorphone and oxycodone were tested. The retention times of these drugs are summarized in Table I.

TFAA derivatives are stable for 48 h, then a degradation product appears in the chromatograms and the morphine and nalorphine peaks are split into two parts.

Clinical data

The proposed method was applied in a bioavailability study and the results are summarized in Table II. The maximum concentration of total morphine occurred between 2 and 4 h after oral administration of Moscontin. The plasma morphine levels were very variable and correlate roughly with the amounts of morphine

TABLE II

CLINICAL DATA AND MORPHINE CONCENTRATION OF PATIENTS TREATED WITH MOSCONTIN (MORPHINE SULPHATE TABLETS)

Case[a]	Concomitant drug therapy	Renal and liver functions	Dose per day (mg)	Analyte[b]	Time after oral administration of Moscontin (h)			
					0	2	4	8
M, 40 yrs, primary cancer unknown	Cis-platinum, 5-fluorouracil, methotrexate, Holoxan, Moscontin (3×230 mg per day)	Normal	3×230	A	3.8	5.4	4.8	4.2
				B	0.5	2.5	0.7	0.4
				C	13.1	21.6	14.6	9.5
M, 29 yrs, lung adenocarcinoma	Cis-platinum, Endoxan, Belustine, Duphalac Persantin moscontin (3×230 mg per day)	Normal	3×230	A	3.5	5.87	4.5	5.63
				B	0.3	0.6	0.4	0 5
				C	8 6	10 2	8.9	8.9
M, 60 yrs, prostate carcinoma	Adriblastine, 5-fluorouracil, Endoxan, Solupred, Kalienor, Lasilix, Moscontin (2×90 mg per day)	Normal	2×90	A	0.37	0.57	0 99	0.73
				B	0.03	0.05	0.09	0.06
				C	8.3	9.5	9	8.2
M, 61 yrs, larynx epidermoid carcinoma	Cis-platinum, 5-fluorouracil, Torental, Efferalgan, codeine, Moscontin (2×70 mg per day)	Normal	2×70	A	0.32	0.34	0.73	0.48
				B	0.03	0.07	0.07	0.03
				C	9.4	20.5	9.6	6.25
F, 44 yrs, ovarian carcinoma	Solupred, Tranxene, Survector, Tenormine, Duphalac, Moscontin (2×30 mg per day)	Chronic renal failure, creatinine = 389 μmol/l, urea = 60 mmol/l	2×30	A	1.375	1.710	0.915	0 475
				B	0.120	0.150	0.09	0 03
				C	8.7	8 8	9.8	6.3

[a] M = males; F = females.

[b] A = Total morphine (μg/l), B = free morphine (μg/l); C = free/total morphine ratio

administered when renal function was normal. Urinary morphine excretion plays an important part in morphine elimination; chronic renal failure greatly increases plasma morphine concentrations and the drug toxicity. The concentration of free morphine paralleled that of total morphine. The free morphine represented about 10% of total morphine, which is in good agreement with other results [11].

DISCUSSION

The use of morphine in the treatment of severe pain in cancer patients is increasing, hence an accurate check of morphine plasma concentrations is needed in order to investigate the relationship between pain control and morphine plasma level.

We developed a GC method using a recently marketed macrobore column. This column is easy to handle, the injection is on-column, it allows the rapid assay for morphine (the retention times of morphine and nalorphine are 10.54 and 11.78 min, respectively) and it has good efficiency and selectivity. The selectivity is improved by the use of a nitrogen–phosphorus-selective detector. The selectivity of the method was checked by mass spectrometry. In all the plasma samples from morphine-treated patients, no compound was found to interfere with the peaks of morphine and the internal standard. However, as each patient received various drugs (Table II), we also checked that drugs such as pethidine, methadone, dextromethorphan, codeine and heroin did not interfere with the assay of morphine.

The extraction procedure has a good recovery and efficiency. However, the recovery is slightly lower than that obtained with Sep-Pak C_{18} cartridges [6] or an Extrelut column with chloroform–isopropyl alcohol as solvent [12]. The use of an Extrelut column reduced the volume of solvent necessary for liquid–liquid extraction. Enzymatic hydrolysis in combination with a simple extraction procedure furnishes very clean plasma samples [13]. As can be seen in Fig. 2, no peak in drug-free plasma overlaps within that of morphine or nalorphine (more than twenty plasma samples were tested).

The good sensitivity of this method allows free and total morphine plasma levels in clinical samples to be monitored. However, free morphine levels are at the limit of detection in some samples. This problem can be overcome simply by increasing the volume of plasma initially extracted.

We investigated five cancer patients treated orally with Moscontin (flow release formulation). The maximum level of morphine was reached between 2 and 4 h after oral administration and then decreased slowly. The plasma morphine concentrations observed were similar to those evaluated by other workers after morphine hydrochloride had been administered orally, but the maximum concentration was greater and the return to baseline values was more rapid with morphine hydrochloride [6]. These results can help physicians to adjust the frequency and time of drug administration. The plasma morphine concentrations roughly parallel the amounts of morphine administered, but the renal elimination and enterohepatic cycle recirculation play an important role in plasma morphine levels. The pharmacokinetics of morphine have not been fully elucidated and a

study undertaken in rats has shown that morphine is detected in body organs 22 days after abrupt withdrawal [9]. The storage of morphine in human body organs and its release in chronic treatment are not well understood.

It can be concluded that the GC method developed here is suitable for the determination of free and total morphine in plasma and for pharmacokinetic, chronopharmacological [14] and metabolic studies of the new biopharmaceutical forms of morphine.

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