Journal of Chromatography, 309 (1984) 73—80
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2115

DETERMINATION OF MORPHINE IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

EVIDENCE FOR PERSISTENT TISSUE BINDING IN RATS TWENTY-TWO DAYS POST-WITHDRAWAL

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(First received October 20th, 1983; revised manuscript received February 16th, 1984)

SUMMARY

Combined gas chromatography—mass spectrometry with capillary and packed column gas chromatography and a deuterium-labelled internal standard was used to determine morphine in biological specimens from rats 22 days after abrupt withdrawal. Morphine was extracted from urine and body organs at pH 9 and the pentafluoropropionyl derivatives were made for analysis by gas chromatography—mass spectrometry. The stationary phase was OV-17 and the mass spectrometer was focused on *m/z* 414 for morphine and *m/z* 417 for the internal standard, [NC^2H_3]morphine. With fused-silica capillary columns, the sensitivity of the assay was increased about ten-fold over packed columns. Urinary excretion of total morphine (free + conjugated) was 22 ng/h (range 11—51 ng/h, $n = 8$) at 22 days post-withdrawal. Free morphine was mainly detected in the lung (1.8—6.5 ng/g, $n = 7$), kidney (1.5—4.0 ng/g, $n = 7$) and liver (1.8—4.6 ng/g, $n = 4$). Traces of morphine were also detected in brain of some rats. Treatment with the opiate antagonist naltrexone, 10 mg/kg on four consecutive days before death, failed to change the urinary excretion pattern or the

0378-4347/84/$03.00

concentration of free morphine in body organs. The biological significance of the residual morphine, if any, remains to be determined.

INTRODUCTION

A host of analytical methods exist for the determination of morphine and its congeners in biological material [1—3]. The combination of gas chromatography and mass spectrometry (GC—MS) with selected ion monitoring furnishes a method with high sensitivity and unsurpassed specificity. This technique has been widely used for the quantitative analysis of drugs of abuse in body fluids and tissue and has emerged as a reference method [4].

We recently showed that rats chronically treated with morphine continued to excrete the drug for several weeks after abrupt withdrawal [5]. This persistence of morphine in the body may have important implications in the withdrawal process and the duration of the abstinence phase. Moreover, if the residual morphine retains its pharmacological activity it could account for the physiological disturbances seen in man and animals during protracted abstinence [6, 7]. The unequivocal identification of free morphine in body organs or tissue from late abstinent rats would support the notion of an in vivo depot of opiate associated with tolerance and dependence.

This paper describes a GC—MS method for the quantitative analysis of morphine by multiple ion detection with a deuterium-labelled analogue as internal standard. This technique was used to detect morphine in the urine and body organs of rats killed 22 days after abrupt withdrawal.

MATERIALS AND METHODS

Drugs and chemicals

Morphine hydrochloride was obtained from the pharmacy at Karolinska Hospital and a deuterium-labelled analogue [NC^2H_3] morphine was synthesised by Mass Analysis (Stockholm, Sweden). Naltrexone hydrochloride was a gift from Endo Labs. (New York, NY, U.S.A.). Pentafluoropropionic anhydride (PFPA) was bought from Mass Analysis and all other chemicals and solvents were of the purest grade obtainable through local suppliers (Kebo Grave, Stockholm, Sweden).

Morphine and natrexone were dissolved in 0.9% (w/v) sodium chloride to make solutions in the concentration range 0.5—3.0% (w/v) of the opiate. For constructing calibration curves and for use as internal standard, morphine and its deuterium-labelled analogue were dissolved in ethanol and calculated as nanogram of the free base.

Animals and treatment

Male Sprague—Dawley rats (Anticimex, Sollentuna, Sweden) were used as animal model of morphine addiction. They were chronically treated with morphine by twice daily (9.00 and 18.00 hours) intraperitoneal injections with gradually increasing doses. The first dose was 10 mg/kg body weight, which was maintained for two days, then 20 mg/kg (two days) and thereafter 20 mg/kg

increments every second day to a maximum of 200 mg/kg. Rats in a control group were injected with 0.9% sodium chloride.

Morphine withdrawal and abstinence

Rats were abruptly withdrawn from morphine after 22 days of chronic treatment (10—200 mg/kg). After seven days of abstinence the animals were moved to metabolic cages and their daily urine samples were collected for the following fourteen days. The samples were frozen at −20° C until analysed. At 18, 19, 20 and 21 days post-withdrawal one group of abstinent rats (n = 7) was injected with 10 mg naltrexone per kg body weight. Another group of morphine-abstinent rats received injections of 0.9% (w/v) sodium chloride.

At 22 days post-withdrawal all the animals were killed by decapitation after a few seconds of anaesthesia with chloroform. Body organs (brain, lung and kidney) were rapidly dissected out, washed free of blood and extraneous tissue fragments and frozen at −20° C pending analysis. The liver from some of the rats was also analysed for morphine.

Extraction and analysis of morphine

Total morphine (free + conjugated) was determined in urine whereas only free morphine was determined in body organs. Two slightly different procedures were employed for the clean-up of biological samples.

Samples of urine (0.1—1 ml) were transferred into polypropylene test tubes and diluted to 2 ml with water and/or hydrochloric acid to give a final acidity of 1 *M*. Thereafter, 50—200 ng of [NC^2H_3]morphine were added as internal standard; the exact amount depended on the concentration of morphine expected from the results of previous determinations. After mixing, the samples were autoclaved at 110° C for 30 min to hydrolyse morphine glucuronide. The samples of urine were allowed to cool and the pH was adjusted to 9 with ammonium hydroxide—ammonium chloride buffer to give a total volume of about 4 ml.

The buffered aliquots of hydrolysed urine were extracted with organic solvent (chloroform—isopropanol, 3:1) by mixing in a shaking table device for 15 min. After centrifuging at 1400 *g* for 10 min, the organic solvent phase was transferred to a new polypropylene tube containing 1 ml of 1 *M* hydrochloric acid. The morphine was back-extracted into the aqueous phase and the solvent was discarded. Finally, the aqueous sample was adjusted to pH 9 and the morphine was once again taken into the organic solvent as described above. The solvent phase was moved to a clean glass tube with tapered bottom and evaporated to dryness under a stream of nitrogen at 50° C.

Body organs were thawed at 4° C and quickly homogenized in 5 ml of ice-cold perchloric acid (0.2 *M*) with an Ultra-Turrax blender fitted with a PTFE pestle. To a 3-ml aliquot of the homogenate, 6 ng of [NC^2H_3]morphine were added as internal standard. The pH was then adjusted to 9 with ammonium hydroxide and bicarbonate buffer and the free morphine was extracted by mechanically shaking for 15 min with a mixture of toluene—2-butanol (80:20). After centrifuging, the organic solvent phase was transferred to another tube containing 0.5 ml of 0.05 *M* sulphuric acid and mixed by shaking for 15 min; thereafter the organic phase was discarded. The

acid aqueous phase was buffered to pH 9 and shaken with 4 ml of dichloromethane—2-butanol (80:20) for 10 min. The organic phase was finally transferred to a small glass test tube and evaporated to dryness under nitrogen.

These two somewhat different extraction procedures for the clean-up of biological samples reflect the techniques currently used in our respective laboratories. Neither method is new; and the triple extraction procedure — first into organic solvent then into an aqueous phase and back into solvent — recovers about 60—80% of morphine [8]. The actual recoveries with [^{3}H]-morphine as marker were 64.8 ± 3.8% (± S.D., n = 5) with tissue homogenates and 64.9 ± 2.4% with specimens of urine. Because we added deuterated morphine as an internal standard no corrections were made to allow for less than 100% recovery.

Gas chromatography

For the GC analysis we prepared pentafluoropropionyl derivatives of morphine. PFPA (100 μl) was added to the residue after evaporation of solvent. The tubes were sealed with tight-fitting stoppers and placed in a heating cabinet at 60° C for about 30 min. The remaining PFPA was evaporated under a stream of nitrogen and the residue was dissolved in ethyl acetate (50 μl) for GC analysis. These PFPA derivatives of morphine were stable for about one week when kept in a refrigerator at 4° C.

The packed column was made from a silanized glass tube (130 cm × 3 mm I.D.) with OV-17 as the stationary phase. The oven temperature was 250° C, injection port 280° C; helium was the carrier gas at 20 ml/min. A 1—2 μl sample was injected and the retention time of morphine was about 2.8 min. The capillary column was made of fused silica (25 m × 0.3 mm I.D.) with OV-17 as the stationary phase. Column temperature was 270° C, and helium was used as carrier gas. A droplet (1—4 μl) of the sample was transferred to the tip of a movable glass needle, and after evaporation of the solvent the needle together with the sample was moved down to the column. The retention time of morphine under these conditions was about 1.5 min. Westerling et al. [9] recently reported a GC—MS assay for morphine in plasma samples employing glass capillary columns.

Mass spectrometry

An LKB 2091 combined gas chromatograph—mass spectrometer was used for the assay of morphine in specimens of urine. The operating conditions were separator 240° C, ion source 270° C, trap current 50 μA, and energy of the electrons 22.5 eV. The concentration of free morphine in body organs was determined with a Finnigan 4000 GC—MS unit and an Incos 2300 data system for data collection. The instrument was operated in the electron impact mode. The other operating conditions were ion source 250° C, electron emission current 0.3 A, and energy of the electrons 40 eV. The electron multiplier voltage was set at 2500 V.

The electron impact mass spectrum of the PFPA derivatives of morphine has a base peak at m/z 414 and a molecular ion with 30% relative intensity at m/z 577. For quantitative analysis the multiple ion detector was focused on m/z 414 for morphine and m/z 417 for the [NC^2H_3]morphine internal standard. In

some runs, the molecular ions at *m/z* 577 and *m/z* 580 were monitored in addition to the base peaks.

RESULTS

Validity of the GC—MS assay of morphine

Linear calibration lines were obtained when the peak height ratio *m/z* 414/417 was plotted against concentration of morphine in spiked standard samples. At the high concentration range (0—200 ng/ml) with a packed column, the regression equation was $Y = 0.025 + 0.005X$ (correlation coefficient, $r = 0.99$) and with the capillary column (0—20 ng/ml) $Y = 0.027 + 0.056X$ ($r = 0.99$). The intercepts on the ordinate can be attributed to the presence of about 2% of non-deuterated morphine contaminating the internal standard. Even at the zero level, when the [NC^2H_3]morphine was added as internal standard a small background peak appeared at *m/z* 414.

Typical mass fragmentograms are shown in Fig. 1, representing a run made with the Finnigan GC—MS with data collecting system. The biological specimen was kidney and the *m/z* 414 peak corresponds to about 4.5 ng of morphine in this organ. The occurrence of mass fragments at *m/z* 414 and *m/z* 417 with very close retention times on this relatively clean chromatogram strongly supports the presence of morphine. The deuterated compound elutes slightly earlier than the unlabelled species.

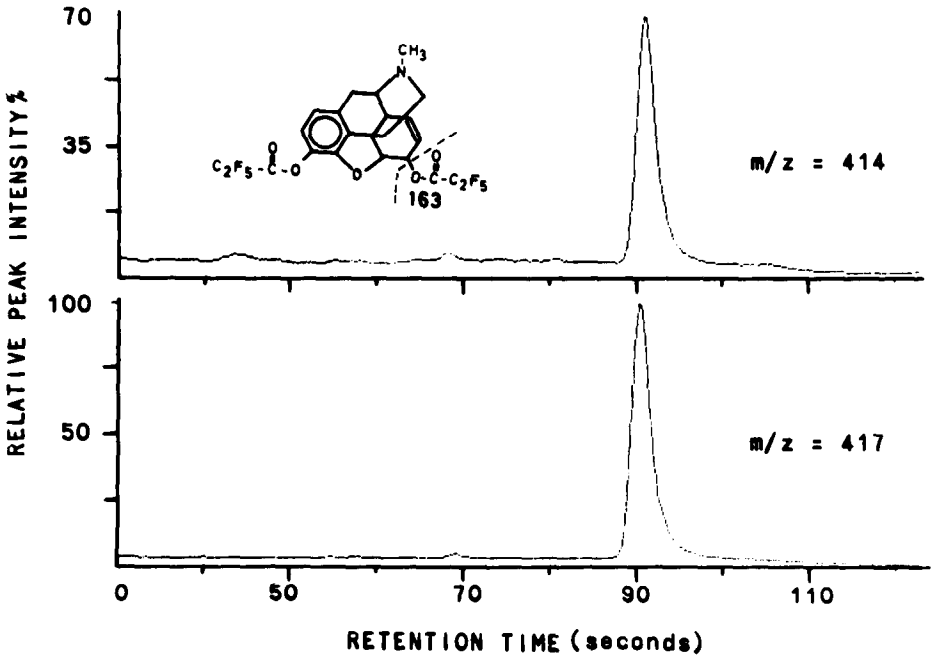

Fig. 1. Typical mass fragmentograms obtained from the assay of morphine by GC—MS (Finnigan Model 4000) with data system. The mass fragments at *m/z* 414 (free morphine) and *m/z* 417 (deuterium-labelled internal standard) show almost identical retention times. The structure of the pentafluoropropionic anhydride derivative of morphine (molecular weight = 577) used for the analysis is shown.

The precision of this method of analysis was assessed (A) from the variance of differences between duplicate determinations and (B) from spiked tissue homogenates. From duplicate determinations at 100 ng/ml urine, the coefficient of variation (C.V.) was 5% and at 10 ng/ml it was nearer 10%, implying a lower precision. Spiked tissue homogenates with 1 ng and 20 ng of morphine resulted in coefficients of variation of 10.8% and 2.1%, respectively (n = 5). At these low concentrations of morphine, the risk of loss of sample during extraction and GC—MS analysis is diminished by the presence of the deuterated internal standard.

Urinary excretion of morphine

Fig. 2 shows the excretion profiles of morphine in individual rats for samples of urine collected between 7 and 22 days post-withdrawal. Total morphine is plotted on the ordinate because samples were hydrolysed before the assay. In spite of fairly wide variations among the rats, a similar excretion time course is evident. The peaked character of some of the curves in the early stage of sampling is difficult to explain. At 22 days, the median excretion of morphine was 22 ng/h (range 11—51 ng/h, n = 8). In a preliminary kinetic analysis of these curves the half-life of morphine excretion for this terminal period, at 18—22 days post-withdrawal, was estimated at ten days [5]. In a group of rats treated with naltrexone a smilar pattern of morphine excretion was noted (data not shown).

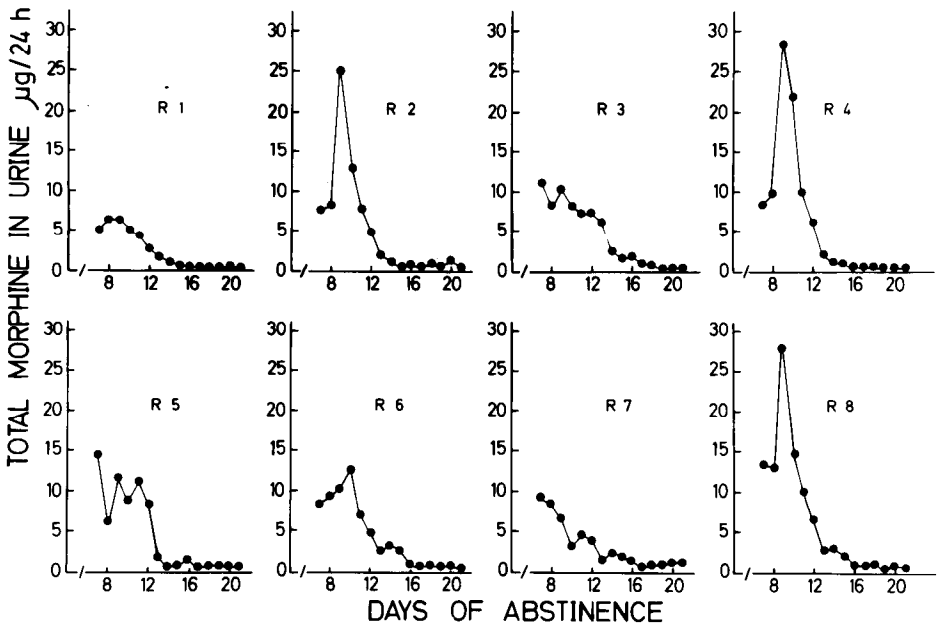

Fig. 2. Urinary excretion profiles of morphine in eight individual rats between 7 and 22 days after withdrawal. Total morphine (free + conjugated) was assayed.

Morphine concentrations in body organs

Table I gives the concentrations of morphine determined in kidney and lung of rats killed 22 days after abrupt withdrawal. The levels were well above the

TABLE I

CONCENTRATIONS OF FREE MORPHINE IN KIDNEY AND LUNG OF RATS KILLED 22 DAYS AFTER ABRUPT WITHDRAWAL

One group of morphine-abstinent rats was injected with 10 mg/kg naltrexone on four consecutive days before they were killed. A control group received injections of saline.

Body organ	Treatment	Concentration of morphine (ng/g)		
		Median	Range	*n*
Kidney	Saline	2.5	1.5—4.0	7
	Naltrexone	3.6	0.6—8.2	7
Lung	Saline	2.6	1.8—6.5	7
	Naltrexone	3.0	1.8—18.8	4

background detection limits (0.2 ng/g). Injections (intraperitoneal) of naltrexone did not change this distribution pattern of morphine. The liver contained 1.8—4.6 ng/g morphine (n = 4); the brain contained 1.0—4.6 ng/g (n = 6), but the levels were below detection limits in the other rats tested.

DISCUSSION

In our previous study on morphine excretion in abstinent rats the concentrations of free morphine in tissue and body organs were near the borderline of the limits of detection. This early study was hampered by the limited sensitivity associated with the use of packed columns for GC—MS assay, as well as background from the deuterium-labelled analogue used as internal standard. In the present method, fused-silica capillary columns furnished a roughly ten-fold increase in sensitivity and even trace quantities of morphine were readily detectable in the body organs.

The distribution pattern of residual morphine in late abstinent rats fits with the results from acute administration of morphine [10]. The kidney contained most free morphine, as might be expected, because rats excrete substantial amounts of morphine as the base [11]. Free morphine in the liver could have been the result of enterohepatic circulation as suggested by others [12]. Even the lung can take part in metabolism of morphine to its glucuronide, and free morphine might therefore accumulate in lung tissue.

The excretion of morphine in urine was assessed as total morphine (free + conjugated) making it difficult to compare results with the levels of free morphine determined in kidney. The peaks seen in Fig. 2 might have resulted from a higher muscular activity of the rats on these days because they were regrouped together for the purpose of cleaning the metabolic cages. They had previously been housed individually for the first week of abstinence. On mixing, rats were highly active and ran around their cages; vigorous fighting broke out among some animals.

With the use of highly specific GC—MS methodology and deuterium-labelled internal standard it seems that the presence of morphine in abstinent rats is definitely established. But what is its biological significance? This is the crucial question that remains unanswered in this study. It seems unlikely that the

residual morphine remains pharmacologically active because injections of naltrexone, a specific opiate antagonist, failed to precipitate any of the well known signs of abstinence. A non-specific binding to tissue components and anionic groups is the most likely explanation.

Both humans and rats have a protracted abstinence phase associated with opiate withdrawal that can last for several months. During this period of withdrawal subtle physiological disturbances persist and addicts undergoing rehabilitation often tend to relapse to using narcotics [13]. Speeding up the excretion of residual morphine might prove a worthwhile treatment but naltrexone showed no clear-cut effects in this connection. Other opioid antagonists with affinity for subpopulations of receptors were not tested.

This study, making use of rats as an animal model of morphine dependence and abstinence, further documents the long wash-out constant of morphine in protracted abstinence [14, 15]. Additional investigations are needed to establish the significance of the residual morphine, if any, in the opiate withdrawal syndrome.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council, the Swedish Delegation for Social Research and the Karolinska Institute Fund.

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