Simultaneous Quantitation of Morphine, Monobutanoylmorphine and Dibutanoylmorphine Using Short Capillary Column Gas Chromatography - Mass Spectrometry

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An assay procedure for quantitating 3,6-dibutanoylmorphine, a morphine diester, and its metabolites, 6-monobutanoylmorphine and morphine, has been developed. The method involves a one-step extraction from various biological media, gas chromatographic separation using a short methylsilicone capillary column and sensitive and selective quantitation by single ion monitoring mass spectrometry. The use of short capillary column GC - MS allowed the rapid separation of compounds with dissimilar retentions that are biologically interrelated and must therefore be measured intact in small sample volumes. The application of this method to studies on the metabolism of butanoyl esters of morphine in whole brain homogenates is described.

Keywords: *Dibutanoylmorphine; morphine esters; gas chromatography; mass spectrometry*

3,6-Dibutanoylmorphine (DBM) is a semi-synthetic diester derivative of morphine that has been previously shown to be an opioid agonist *in vitro*,[1] to act as a potent analgesic *in vivo*[2] and to produce less severe behavioural effects than other morphine esters when administered to rats.[3] Investigation of the regional metabolism and disposition of DBM required a method of reliably and rapidly quantitating unchanged DBM and its primary metabolites, 6-monobutanoylmorphine (MBM) and morphine (M), in a variety of tissues and tissue homogenates.

A number of assay procedures have been described for the quantitation of morphine using high-performance liquid chromatography (HPLC) coupled to electrochemical,[4–6] ultraviolet[7] or fluorescence[8] detectors; however, these methods either lacked the required sensitivity or were incapable of measuring unchanged DBM. Similarly, radioimmunoassay (RIA) methods for measuring morphine concentrations have been described,[9–11] but these lack the necessary specificity for distinguishing between such chemically similar compounds as M, MBM and DBM.

The technique of gas chromatography - mass spectrometry (GC - MS) seemed the most appropriate method for the current application as this technique has been successfully applied to the quantitation of morphine[12,13] and allows the detection of DBM without either derivatisation or hydrolysis. The procedure described in this paper involved a one-step extraction procedure for all three compounds (M, MBM and DBM), a rapid chromatographic separation using a very short methylsilicone capillary column and a highly selective method of detection using selected ion monitoring (SIM) mass spectrometry.

Experimental

Equipment

The assay of M, MBM and DBM was conducted using a Hewlett-Packard (Palo Alto, CA, USA) 5985 GC - MS system operated in the electron-ionisation (EI) mode. Default tuning parameters for the mass spectrometer were established using the program Autotune and perfluorotributylamine as the calibration standard at a source pressure of approximately 2×10^{-6} Torr. The mass spectrometer was operated at an ion source temperature of 200 °C and the electron multiplier voltage was increased by 600 V over the default voltage.

Drugs and Reagents

Morphine sulphate was purchased from BDH (Toronto, Ontario, Canada). MBM was synthesised as the hydrochloride salt using the method of May and Jacobson[14] adapted for butanoyl derivatives. DBM was synthesised according to the method of Beckett and Wright[15] and was converted into the hydrochloride salt. MBM and DBM were authenticated by mass spectrometry.

Ethyl acetate, chloroform, butan-1-ol and acetonitrile were HPLC-grade solvents from Fisher Scientific (Whitby, Ontario, Canada). Pyridine and acetic anhydride were of spectrophotometric grade, from Fisher, and anhydrous potassium phosphate (dibasic) was obtained from J. T. Baker (Phillipsburg, NJ, USA).

Homogenate Preparation

Male Sprague-Dawley rats (200–300 g) (Canadian Breeding Farm Laboratories, Montreal, Quebec, Canada) were killed by decapitation and the brains were removed on ice. Whole brains were homogenised in cold Dulbecco's phosphate-buffered saline (pH 7.35) to produce 10% *m/V* homogenates after addition of drug. Homogenates containing drug were incubated at 37 °C in a shaking water-bath (Precision Scientific, Chicago, IL, USA).

Extraction and Derivatisation Procedure

Samples (0.5 ml) of 10% *m/V* whole brain homogenate were removed at selected time points and mixed with 0.5 ml of 100 mM K_2HPO_4 - acetonitrile (50 + 50 *V/V*, pH 8.7) in glass test-tubes. Ethyl acetate - chloroform - butan-1-ol (80 + 10 + 10 *V/V*, 2.0 ml) was then added and the mixture was shaken for 5 min in a horizontal shaker (Eberbach, Ann Arbor, MI, USA) and then centrifuged at 250 *g* for 5 min in a bench-top centrifuge (Model HN-S; Damon/IEC, Needham Heights, MA, USA). An aliquot (1.0 ml) of the upper (organic) phase was then removed and evaporated to dryness under nitrogen in a 1.5-ml polypropylene microcentrifuge tube (Sarstedt, St. Laurent, Quebec, Canada).

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M and MBM were derivatised for GC analysis by dissolving the extraction residue in 0.2 ml of pyridine and incubating with 0.5 ml of acetic anhydride for 60 min at 60 °C in a dry bath (Fisher Scientific). Derivatised samples were then dried under nitrogen and were stored at −70 °C until undergoing analysis by GC - MS.

Chromatography

Residues were dissolved in 50 µl of ethyl acetate and an aliquot (1 µl) was injected on to the GC column, which consisted of a 3-m section of methylsilicone glass capillary column (SPB-1, 0.75 mm) (Supelco, Bellefonte, PA, USA). The injection port temperature was 250 °C and immediately following injection a programmed linear temperature gradient was initiated such that the oven temperature increased from 150 to 270 °C at a rate of 12 °C min^{-1}. The carrier gas (helium) was delivered at a flow-rate of 35–40 ml min^{-1}. The GC effluent passed via a jet separator into the ionisation chamber of the mass spectrometer and detection was accomplished using SIM at a mass to charge (m/z) ratio of 268.

Results and Discussion

A chromatogram obtained following extraction, derivatisation and injection of a 0.5-ml brain homogenate sample containing M, MBM and DBM is shown in Fig. 1. The peaks corresponding to the appropriate derivatives (3,6-diacetylmorphine, 3-*O*-acetyl-6-butanoylmorphine and DBM for M, MBM and DBM, respectively) were well resolved and eluted within 7.5 min. Both the column length and the oven temperature gradient were found to be critical for suitable chromatographic separation. DBM has a low vapour pressure and is unstable at temperatures above approximately 300 °C. DBM therefore failed to elute within 20 min using any of a variety of normal 6-m packed columns or a 30-m capillary column at temperatures below 300 °C. Use of the short capillary column, however, resulted in DBM retention times of less than 10 min at these temperatures. Although the peak shapes were acceptable using the short column, a mixture of M, MBM and DBM was inadequately resolved using isothermal chromatography; however, this problem could be suitably overcome by using a programmed temperature gradient. Thus, either a temperature gradient or a short column alone was insufficient for the current application, but these two factors in combination resulted in a rapid and effective separation.

Following derivatisation with acetic anhydride, all three analytes were diester derivatives of morphine; consequently, the mass spctrometer fragmentation patterns were very similar. Examination of the mass spectra revealed that all compounds had a strong ion abundance signal at m/z 268, in accord with results reported previously for other morphine diesters.[1] The use of SIM as a means of detection resulted in both good sensitivity (lower limit of detection ≈ 0.3 ng for M, 0.5 ng for MBM and 0.9 ng for DBM based on a signal to noise ratio ≥ 2) and good selectivity, as no interference at the monitored ion was found in blank samples of either blood or brain homogenate, whereas considerable background noise was found in total ion chromatograms of the GC effluent. Calibration graphs (peak area *vs.* nanograms of drug) for M, MBM and DBM were found to be linear from 10 to 250 ng and passed through the origin. The correlation coefficients for all these drugs were ≥0.9916.

Over-all coefficients of variation (C.V.) following extraction, derivatisation and injection of aqueous standards containing 25 ng each of M, MBM and DBM (n = 6) were 5.3%, 9.4% and 10.2% for M, MBM and DBM, respectively, and mean C.V.s for brain homogenate samples (n = 3) over a range of eight drug concentrations were 10.2%, 9.9% and 13.0% for M, MBM and DBM, respectively. The reproducibility in the current assay is comparable to that of existing GC assays for morphine alone[16,17] and is superior to that achieved in other assays for morphine and morphine derivatives.[5,13] For these reasons the use of internal standards was considered unnecessary for the current applications, especially as the different physico-chemical properties of M, MBM and DBM would require that an internal standard for each compound be incorporated into the assay. Ideally, stable isotope analogues of M, MBM and DBM might have been used to increase the reproducibility of the procedure. Unfortunately, these were not available to us.

The metabolism of a mixture of butanoyl esters of morphine by rat brain homogenates (n = 4) was studied using the assay procedure as described; the relative amounts of DBM, MBM and M in brain homogenates at selected time points are presented in Table 1. The concentrations of morphine esters were not significantly reduced at any time in buffer controls, but DBM was metabolised to MBM and subsequently to M in brain homogenates ($t_{1/2}$ = 69 min). In previous studies on the metabolism of DBM in both rat and human blood, it was found that whereas DBM concentrations decreased exponentially, the metabolism terminated at the level of MBM.[18] Hence brain tissue appears to contain enzymes that can deacylate MBM to M, whereas blood does not. This result is important to an understanding of the actions of DBM within the brain, as both MBM and M, but not DBM, are presumed to be pharmacologically active.[2]

The procedure described in this paper represents a relatively simple and effective method of simultaneously determining three biologically interrelated compounds that have

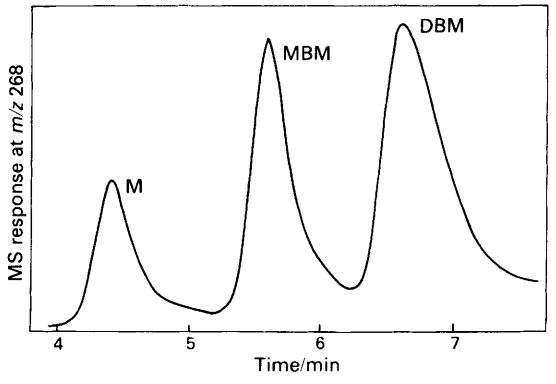

Fig. 1. Chromatogram showing M, MBM and DBM in a sample of brain homogenate that had been incubated with 7.5 × 10^{-4} M DBM and 2.5 × 10^{-4} M MBM for 60 min

Table 1. Biotransformation of a mixture of DBM and MBM by rat brain homogenates. DBM and BMB were added to 10% m/V rat brain homogenates to initial concentrations of 7.5 × 10^{-4} and 2.5 × 10^{-4} M, respectively. At the time indicated, samples were taken for analysis as described under Experimental. Data are presented as percentages of the total opioid concentration ± standard deviation (n = 4); asterisks indicate $p \leq 0.01$ relative to time zero by repeated measure ANOVA

Time/min	Concentration, %		
	DBM	MBM	M
0	75.9 ± 2.9	23.3 ± 2.4	0.8 ± 0.6
5	76.4 ± 3.8	20.8 ± 2.7	2.8 ± 1.2
15	74.0 ± 4.0	19.0 ± 1.9	7.0 ± 2.1
30	62.1 ± 1.7*	25.4 ± 0.9	12.4 ± 1.6*
60	46.4 ± 5.8*	35.1 ± 2.7*	18.4 ± 3.6*
90	35.6 ± 6.8*	39.2 ± 1.7*	25.2 ± 6.7*
120	23.8 ± 6.6*	44.2 ± 4.6*	32.0 ± 11.2*
180	15.7 ± 5.1*	45.0 ± 2.9*	39.4 ± 7.8*

dissimilar chromatographic properties. The use of a very short capillary column allows the chromatographic separation of three chemicals with relatively low vapour pressures, and circumvents the need for harsh chemical treatments that might distort the relative concentrations of these drugs in biological samples. As such, the use of similar chromatographic manipulations may provide a solution to comparable problems in the analysis of other biologically active compounds where small sample sizes and rapid analysis times are priorities.

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