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FAST RELIABLE ASSAY FOR MORPHINE AND ITS METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND NATIVE FLUORESCENCE DETECTION

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SUMMARY

A method for the fast analysis of morphine (M), normorphine (NM), morphine-3- and -6-glucuronides (M3G and M6G) and codeine (C) is described which has the advantages of sensitivity, speed and specificity. Dihydrocodeine and heroin can also be assayed. The method is based on extraction of the opiates from serum, plasma and cerebrospinal fluid using reversed-phase solid-phase extraction columns, followed by reversed-phase high-performance liquid chromatography with native fluorescence detection. The extraction step provides > 95% recovery, and the response of the detection system is linear from 0.5 to beyond 750 ng. The method allows analysis of M, NM, M3G, M6G and C. No other drugs have been found to interfere with the assay. The assay offers a quick, cheap and reliable method of specifically determining morphine and its metabolites, including the potent M6G, from a small sample volume; this will be of advantage to both clinician and basic scientist

INTRODUCTION

Until recently, most studies of the disposition and metabolism of morphine (M) in man relied on the measurement of the drug by radioimmunoassay (RIA) [1]. This technique does not specifically measure M, since the antisera commonly used may cross-react with, among others, morphine-3- and -6-glucuronides (M3G and M6G), codeine (C) and normorphine (NM) [2]. Thus RIA measures 'M-like immunoreactivity' and the result will include a contribution from the cross-reacting metabolites or other compounds. This proportion changes according to the antiserum used, making comparisons between the results from different laboratories difficult. Enzymic conversion of the glucu-

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ronides to M allows determination of M and the sum of M3G and M6G but will not distinguish between the glucuronides [3]. In order to determine M and all its metabolites, specific, separate RIAs need to be carried out. For example, a differential RIA has been recently described which allows separate determination of M, M3G and M6G using three distinct antisera with varying cross-reactivities [4]. The use of simultaneous equations allows determination of each analyte. This method, however, assumes the absence of other cross-reacting species and in this particular assay any codeine present in the sample will cause overestimation of all the analytes, since the antisera cross-react to a great extent with C.

During the past five to ten years, methods for the measurement of M by high-performance liquid chromatography (HPLC) have been introduced, using a combination of electrochemical detection (ED) and UV detection for various metabolites [5–7]. ED alone has been used for M [8]; in this case the enzyme β-glucuronidase was used to convert M3G to M, thus measuring the total sum of M and M3G. M6G was not mentioned. The simultaneous detection of M with NM, C, M3G and M6G using ED is not possible unless more than one electrochemical detector is used due to the differing redox potentials of the compounds. HPLC with pre- or post-column derivatisation of the analytes has also been used [9,10]. Such methods can require long incubation times and do not detect any metabolites without the phenolic hydroxy group or a primary amine, for example M3G and C [9]. Fluorescence detection following HPLC has also been used, for example for the related molecule apomorphine [11]; and following oxidation, strongly fluorescent dimers of M [12] or 6-acetylmorphine [13] can be assayed. These latter methods cannot detect 3-glucuronidated compounds because the dimerisation reaction occurs through the phenolic (3-) position. More complex methods, such as gas chromatography–mass spectrometry (GC–MS) [14] have also been used. Such techniques may give unequivocal results, but are extremely expensive and hence are not commonly available. These analytical problems may be responsible for the current controversy concerning the elimination of M in man and the uncertainty as to the potency of M6G in man [15,16].

We have developed a method combining solid-phase extraction of cerebrospinal fluid (CSF), plasma and serum with analytical HPLC and native fluorescence detection. Fluorescence detection with HPLC has been used before for M and other opioids [17], but not for the analysis of clinical samples, or of all the compounds and metabolites reported here. C alone has been assayed in plasma by HPLC followed by fluorescence detection [18]; M, M3G, M6G and NM have been determined simultaneously by a combination of ED with a conditioning cell and fluorescence (for M3G) [19].

The combination of extraction, HPLC and fluorescence detection at specific excitation and emission wavelengths described here provides reliable identification of the compounds in question with minimal interference from natural unidentified compounds or administered drugs at very low limits of detection.

EXPERIMENTAL

Reagents

HPLC solvents were water, HPLC (far-UV) grade acetonitrile (BDH, Poole, U.K.) and protein-sequencing grade trifluoroacetic acid (TFA) (Sigma, Poole, U.K.). Solvents were filtered and vacuum-degassed using a 0.2-μm Anotop filter (Anachem, Luton, U.K.). Morphine sulphate was obtained from the hospital pharmacy as a solution in sterile saline (6 mg/ml), C and M3G from Sigma and M6G from Salford Fine Chemicals (Salford, U.K.). NM was a gift from Dr. H. McQuay (Oxford, U.K.). Other reagents were Analar grade purchased from BDH.

Extraction

CSF and blood from patients undergoing surgical pain relief procedures were collected in plain or heparinised tubes and centrifuged. Serum and CSF were then frozen at $-70°C$ or extracted immediately as follows. Solid-phase extraction columns (Bond Elut C_2, 500 mg per 3 ml, from Jones Chromatography, Hengoed, U.K.) were placed in a Waters-Millipore (Harrow, U.K.) Sep-Pak cartridge vacuum extraction manifold. These were pre-wetted with 2 ml of methanol and the columns were then equilibrated with 2 ml of 0.05 *M* Tris–HCl buffer, pH 7.5. Sample (1 ml, diluted with 1 ml Tris buffer) was applied to the column, and the column was then washed with 10 ml Tris buffer. Elution was carried out with 50% acetonitrile containing 0.1% TFA (2 ml). The eluate was collected in 5-ml polypropylene tubes (Sarstedt, Leicester, U.K.) and dried using an Edwards (Crawley, U.K.) Modulyo freeze drier equipped with a spin-freeze accessory. Alternatively, we dried aliquots of the samples at 40°C under a stream of nitrogen. The dry eluates were then redissolved in a total volume of 250 μl in HPLC starting solvent and 20–200 μl were applied to the HPLC column.

HPLC conditions

A Beckman 100 controller with two 112 pumps (equipped with a Rheodyne 7125 manual injector and 200-μl loop) was used at a flow-rate of 1 ml/min. The column used was a Spherisorb S5 C_6 (Phase-Separations, Queensferry, U.K.) (150 mm$\times$4.6 mm I.D.) with a hexyl pre-column in a cartridge holder (HPLC Technology, Macclesfield, U.K.). The detector was a Merck-Hitachi F1000 dual monochromator fitted with a 12-μl flow cell; the excitation wavelength was 280 nm and the emission wavelength 335 nm. Gradient elution was carried out at room temperature at a constant flow-rate of 1 ml/min. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in 40% acetonitrile. Gradient elution was carried out as follows: initial conditions were 16% B (i.e. 6.4% acetonitrile) at 1 ml/min. After injection of the sample (20–100 μl), the

system was pumped isocratically for 2.0 min, followed by a gradient of 16–50% B (6.4–20% acetonitrile) over 10 min. When CSF or serum samples were injected, this was followed by a gradient to 100% B (40% acetonitrile) over 2 min to wash the column. After 7 min the system was returned to initial conditions. Detection was by native fluorescence of the compounds using an excitation wavelength of 280 nm and emission at 335 nm. The results were recorded on a chart recorder and integrated using a PC-based JCL6000 16-bit integrator (Jones Chromatography). Raw data were stored on the computer hard disk and archived regularly to allow for reintegration at a later date if necessary.

RESULTS

Fluorescence

M and other opiate agonists such as levallorphan have been previously shown to exhibit strong native fluorescence [17]. All the analytes of interest showed fluorescence when excited at 280 nm. The emission spectra were rather broad, and all centred on 330–340 nm. Naloxone shows no fluorescence [17] and diacetylmorphine (heroin) and dihydrocodeine showed much lower fluorescence than the other five compounds. There was no difference in fluorescence yield between phosphate–sodium dodecyl sulphate (SDS)–acetonitrile HPLC solvents (pH 2.1) and water–TFA–acetonitrile (pH 2.1). Since the HPLC detector had a band width of 10 nm, an emission wavelength of 335 nm was chosen; this provided a good response from all the compounds. M3G and C exhibit the highest response and M6G the lowest, at one third that of M3G. Extensive trials of available HPLC fluorescence monitors showed large differences in sensitivity under the conditions used between similar models. The dual monochromator model used in this study showed the highest sensitivity of those tested.

HPLC system

Several HPLC systems were used in initial trials for this assay, including those previously published which used phosphate buffers, acetonitrile and SDS [6,7]. We found, however, that a simple binary gradient system using water–acetonitrile elution on a reversed-phase hexyl column with TFA ion-pairing gave the most reproducible results. The phosphate–SDS systems gave good separation of M, M3G and M6G, but were less reliable and required a very long re-equilibration time after an acetonitrile gradient had been run to achieve separation of other components. The eluents we used also have the advantage of total volatility, allowing simpler HPLC close-down procedures and also allowing for peaks to be collected and dried for subsequent further analysis. Fig. 1 shows an HPLC trace of elution of commercial pure samples of M, M3G, M6G, NM and C under the HPLC conditions we used. As can be seen, the compounds are well separated and have symmetrical, sharp peaks.

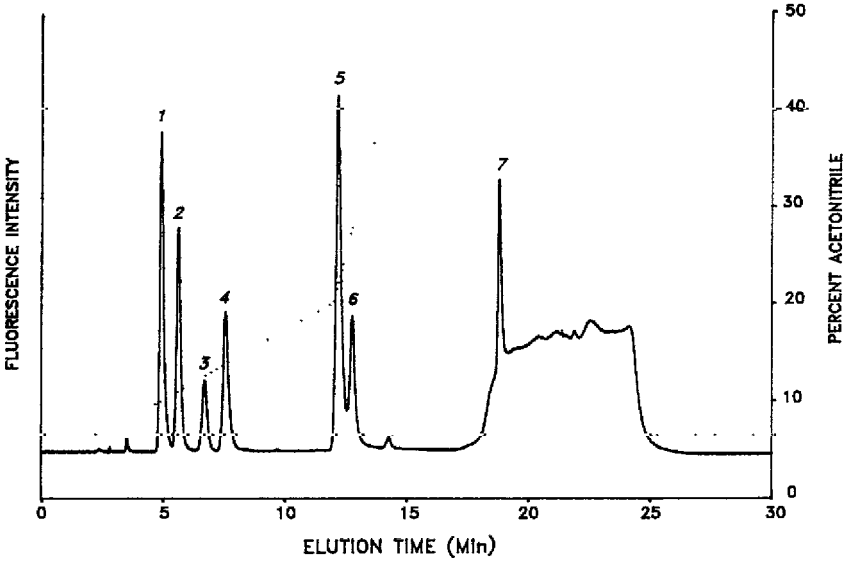

Fig. 1. HPLC–fluorescence trace of opiate standards injected in 100 μl. Conditions as described. Peaks 1 = M3G (400 ng); 2 = NM (400 ng); 3 = M6G (400 ng); 4 = M (400 ng); 5 = dihydrocodeine (2000 ng); 6 = C (400 ng); 7 = heroin (7000 ng). (---) Solvent composition, percentage acetonitrile.

TABLE I

BOND ELUT COLUMN RETENTION CHARACTERISTICS

The columns were treated as described in the text, spiked plasma applied, and eluted samples applied to the HPLC–fluorescence system to determine whether the compounds were retained and subsequently eluted and whether interfering peaks were present.

Column type	Retained analytes	Eluted at 50% acetonitrile	Serum interference
Cn	No	–	–
Diol	No	–	–
C2	Yes	Yes	No
C8	Yes	Yes	Yes
C18	Yes	Yes	Yes
Phenyl	Yes	Yes	Some
CH	Yes	Yes	Yes

Extraction

A range of solid-phase extraction columns was used to identify the column and solvent system that would offer greatest selectivity. Mixtures of the compounds of interest were applied and eluted according to the suggestions of the manufacturer. This involved wetting with methanol, followed by washing with buffer (Tris–HCl, 0.05 *M*, pH 7.4), application of sample in buffer and elution

with methanol–water or acetonitrile–water, in the presence or absence of 0.1% TFA. The results are shown in Table I. The columns were assessed using three stages: loading a mixture of pure analytes (200 ng of each) and determining whether the columns retained them in Tris–HCl buffer at pH 7.5 and whether the compounds could subsequently be eluted by acetonitrile or methanol at 50 and 75%. Those columns that retained the compounds were then assessed for ability to wash off interfering serum substances without loss of analyte, using first serum only, then serum spiked with 200 ng of each analyte. Only the C_2 and phenyl column were suitable under these criteria. There were fluorescent compounds eluting under these conditions after extraction, but those associated with the C_2 column had retention times that did not correspond to any of the compounds being analysed with the exception of C (see below). We also experimented with different wash methods, including the use of dilute aqueous TFA, and different elution protocols. These included various concentrations of acetonitrile and/or methanol (results not shown). The optimum method that gave best recoveries with least interference from non-opiate compounds was that described with a 10-ml wash of Tris–HCl buffer and elution with 50% acetonitrile–0.1% TFA. Fig. 2 shows an HPLC trace of human serum spiked with 400 ng of each compound, as well as unspiked serum, extracted and run

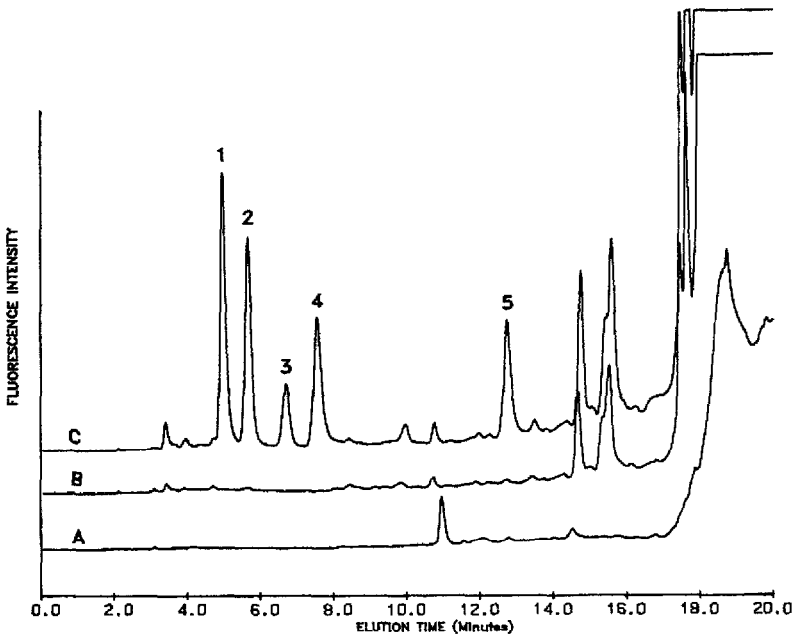

Fig. 2. HPLC–fluorescence traces of (A) extracted blank CSF, (B) extracted blank plasma and (C) blank plasma spiked with 400 ng each of M3G (1), NM (2), M6G (3), M (4) and C (5), extracted. In each case 1 ml was extracted, the dried eluate resuspended in 250 μl, and 100 μl were injected onto the HPLC system.

as described above. Also shown are typical results obtained with blank CSF, i.e. a sample from an M-naive patient. It can be clearly seen that there are no interfering peaks in the unspiked samples except in the case of C in plasma. A small peak coeluting with C is occasionally present, corresponding to approximately 15 ng C. A number of other unidentified fluorescent peaks do appear, mostly eluting later than the opiates.

Recoveries

The recovery of M and the metabolites through the extraction and HPLC system was determined by spiking human serum with a serially diluted mixture of all the compounds under study. These spiked samples (1 ml) were then extracted and analysed as described above and compared with standard HPLC runs using the same mixtures of opioids and metabolites. Fig. 3 shows the recoveries for M, M3G, M6G, NM and C through the system. It can be seen that in the range 15–1000 ng, the recoveries for all the compounds are greater than 95%. Recoveries for C are higher than 100% in the lower concentrations, due to co-elution under these gradient conditions of a small, unidentified peak. Standard errors were in every case less than 5%. When the data were analysed by linear regression, the response of the system to spiked plasma was a straight-line calibration curve. Correlation coefficients were 0.9997 (M), 0.9998 (M3G), 0.9999 (M6G) and 0.9994 (NM). This was for the complete extraction and analysis system from spiked plasma.

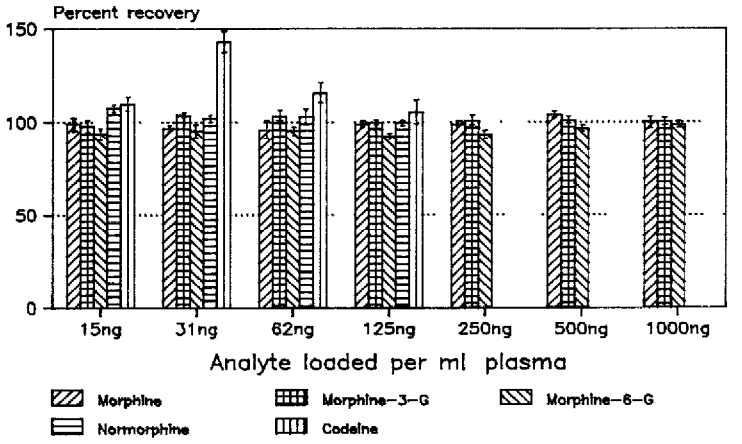

Fig. 3. Recoveries through extraction system. Blank plasma (1 ml) was spiked in triplicate with a mixture of M and metabolites in the range 15-1000 ng. These were then extracted, dried, resuspended and analysed by HPLC with fluorescence detection as detailed in the text. Recoveries are expressed as percentages of original material applied. Standard errors of recovery are shown; in all cases these were less than 5%.

Limits of detection

Under optimum conditions and using commercial samples, the detection limits through the HPLC system are as shown in Table II. The table also shows the detection limits when samples of CSF or plasma are analysed. When such clinical samples are under analysis, the detection limit has to be related to the sample volume used. In this system, the recovery of the glucuronides from the Bond Elut C_2 columns showed a dependence on the volume applied to the column; thus increasing from a 1- to a 2-ml sample (4 ml applied to the extraction column) the recovery for M3G (100 ng) dropped from 99.6 ± 3.0 to $61.0 \pm 6.0\%$ and for M6G from 96.5 ± 4.0 to $47.3 \pm 7.2\%$. Thus the maximum sample volume that could be applied to the extraction column was 2 ml, and the maximum volume of serum or CSF sample was 1 ml. It would be possible to dilute the sample to a lesser extent, thus increasing the sensitivity by a factor of up to 2. The detection limits of the method in the clinical situation, based on 1-ml samples and on the smallest area our equipment could reliably integrate, was as shown in Table II.

TABLE II

LIMITS OF DETECTION IN THE CLINICAL SITUATION AND ON-COLUMN

The clinical detection limit is based on the minimum reliably integrable area with our equipment and a sample volume of 1 ml of CSF or plasma. Limits on-column refer to commercially pure samples applied directly to the HPLC system and analysed under ideal conditions.

Compound	Clinical detection limit		On-column	
	ng/ml	pmol/ml (n*M*)	ng	pmol
M	0.87	2.60	0.32	0.94
M3G	0.52	1.13	0.18	0.39
M6G	1.34	2 90	0.59	1.28
NM	0.59	2.17	0 23	0.84
C	1.11	3.71	0.43	1.20

Interference

As discussed above, we have found no interference from endogenous compounds using this method with CSF, serum or plasma, except for the small peak occasionally present which co-elutes with C. In our case, this was not important, since we do not assay patients receiving C, and have found no evidence for the presence of C in the samples we have analysed. Patients receiving M are unlikely to be receiving C also. We have synthesised codeine glucuronide biochemically using bovine glucuronyl transferase (data not shown) since we do not have access to commercial samples of this metabolite. On our HPLC system, this synthesised codeine glucuronide elutes at 4.25 min, well separated from any of the other compounds of interest. We do, however, find an uniden-

tified peak eluting very close to NM in some samples. This only occurs in samples that have circulating M and metabolites and is thus related to M; this could lead to an overestimation of NM if the separation between NM and the unidentified peak is not to the baseline. On the basis of our biosynthetic experiments, this may be morphine-3,6-diglucuronide. In some samples we found unidentified M-related peaks from patients receiving M, in particular some eluting late in the chromatogram that increase with time after an oral dose in children, and we are attempting to identify these. We have so far found no interference from administered drugs in clinical samples. The drug history of every patient whose serum or CSF we have assayed for M and metabolites is known, and no drug so far tested has interfered with the assay. These drugs include atracurium besylate, atropine, dothiepin, domperidone, dextromoramide, naproxen, ranitidine, thioridazine, trimeprazine and valproate.

We have also compared the results obtained from plasma and serum from the same patient taken at the same time: blood was collected either in lithium heparin or plain vacutainers, centrifuged and assayed as described above. The results were within 3% of each other.

DISCUSSION

Since it has recently been reported that at least one of the metabolites, M6G, is pharmacologically more potent than M itself [15], and that M6G has been used clinically [16], the need for a quick, reliable assay of all the major metabolites and the parent compound has become even more pressing. Fast, sensitive and cheap assays for M and its metabolites have been difficult to achieve as discussed above. This method is highly sensitive, giving sensitivities of less than 1 ng/ml, and results can be obtained in less than 1 h.

HPLC methods require a means of extracting the analytes from the sample matrix and double Sep-Pak cartridge extractions have been used [6] as well as liquid–liquid extraction methods. We have used a single Bond Elut C_2 cartridge which can be re-used at least three times, thus cutting the cost of the assay considerably. The use of each compound's native fluorescence for detection does away with the need for pre- or post-column derivatisation, or the use of the complex ED with two separate detectors.

This method, with its speed and specificity, allied to the fact that M, M3G, M6G and C can be assayed from one sample in a fraction of the time necessary for RIA methods should allow clinicians fast decision-making when faced with patients who are not responding normally to M analgesia. It is a robust assay, unaffected by other drugs or sample presentation. Plasma and serum give identical results, indicating that sample matrix adsorption does not hinder the extraction procedure. We are currently using the method for the analysis of CSF and serum from chronic pain patients receiving slow-release or intrathecal M, as well as in children receiving oral M pre-operatively.

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