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Note

An improved method for the simultaneous determination of morphine and its principal glucuronide metabolites

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Recent awareness of the role of morphine metabolites in the clinical activity of morphine has highlighted the importance of determining both morphine and morphine metabolite behaviour in vivo [1–4]. There is a clear need for a sensitive, specific method for the determination of morphine and its principal metabolites in body fluids.

Such a method has been described by Svensson et al. using solid-phase sample purification, and quantitation by high-performance liquid chromatography (HPLC) using UV detection [5] or electrochemical detection [6].

However, the method of sample purification is time-consuming and cumbersome and an interfering peak eluted close to morphine which was not removed by the sample purification procedure. Refinements to the method permitting easier and more rapid sample preparation and complete removal of the interfering peak giving more reliable sensitivity would be of value. Improvements to this method using an additional screening electrode for electrochemical detection, the use of morphine-6-glucuronide as a reference standard and the use of fluorescence detection for quantitation of the 3-glucuronide of morphine, which is not electrochemically active, are reported here. The use of a microprocessor-controlled extraction device to facilitate simultaneous extraction of multiple samples is also described.

EXPERIMENTAL

Materials

The chromatographic equipment consisted of an Applied Chromatography Systems Model 351 pump (Macclesfield, U.K.) with an ESA Coulochem electro-

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chemical detector (Bedford, MA, U.S.A.) having both a 5021 conditioning cell and a 5011 high-sensitivity cell in series. Eluent was then passed into a Kratos Spectroflow 980 fluorescence detector (Liverpool, U.K.) with a deuterium source operating at an excitation wavelength of 210 nm with a 300-nm cut-off filter on the emmision. Sample introduction was by means of a Rheodyne valve incorporated in a Perkin-Elmer ISS-100 autoinjector (Beaconsfield, U.K.).

Morphine-3-glucuronide was obtained from Sigma (Poole, U.K.) and morphine-6-glucuronide specially synthesised by Salford Ultrafine Chemicals (Chemistry Tower, Salford University, Salford, U.K.). The acetobromo sugar derivative necessary for the synthesis of the morphine-6-glucuronide was kindly provided for the initial synthesis by Dr. Yoshimura (Kyushu University, Fukuoka, Japan).

Sample purification

Sample purification was carried out as described by Svensson et al. [5] using Sep-Pak C_{18} cartridges (Millipore-Waters, Harrow, U.K.), which were conditioned with 5 ml of methanol, 3 ml of 10 m*M* sodium dihydrogenphosphate (pH 2.1) containing 10% acetonitrile and 5 ml of water. A 1-ml volume of plasma, buffered with 3 ml of 500 m*M* ammonium sulphate (pH 9.3), was applied to the cartridge and after washing with 20 ml of 5 m*M* ammonium sulphate (pH 9.3), followed by 0.5 ml of water, morphine and its metabolites were eluted with 3 ml of 10 m*M* sodium dihydrogenphosphate (pH 2.1), containing 10% acetonitrile. This extract was then buffered with a further 3 ml of 500 m*M* ammonium sulphate and treated on a second cartridge in exactly the same way. A 1-ml volume of this eluent was injected onto the HPLC column.

Multiple extraction device

Because of the slow, cumbersome nature of individual sample preparation (which may take up to 7 min for each sample), a device was constructed to allow the simultaneous extraction of up to five samples, as shown in Fig. 1. This device

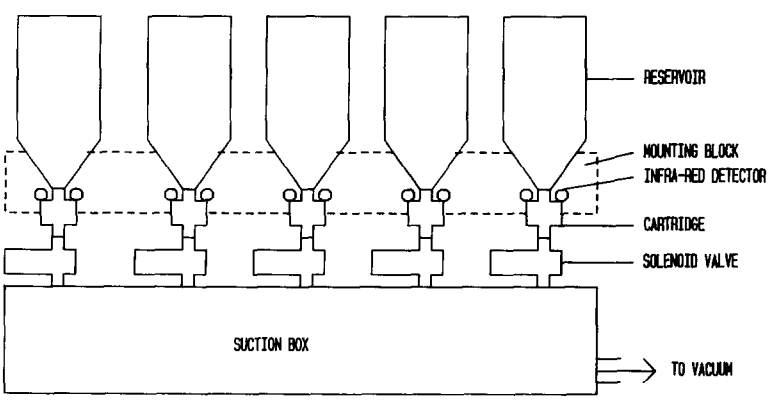

Fig. 1. Schematic representation of the multiple extraction device with individual cartridge control described.

automatically stops flow in individual cartridges when the fluid level in the reservoir reaches the top of the cartridge. The top of fluid is detected by an infrared sensor which initiates closure of the solenoid valve via a microprocessor control unit. Extraction of samples using this system was compared with a currently available multiple extraction device without individual cartridge control (Millipore-Waters) and with extraction of samples individually. In each case negative pressure was applied to the cartridges by means of a vacuum pump.

Chromatography

Chromatography conditions were as described by Svensson et al. [5], except that an Apex ODS column (150 mm×4.6 mm) was used (Jones Chromatography, Llanbradach, U.K.). The mobile phase consisted of 10 m*M* sodium dihydrogenphosphate (pH 2.1), 1 m*M* sodium dodecyl sulphate and 26% acetonitrile and was filtered through a 0.45-μm filter before use.

RESULTS

Extraction

Reproducibility of extraction, using each of the devices described, is given in Table I. Using the multiple extraction device, the extraction efficiency was >80% for morphine and morphine-6-glucuronide at levels of 20, 80 and 400 ng/ml, and >80% for morphine-3-glucuronide at levels of 200, 800 and 4000 ng/ml with coefficients of variation of <6% in each case. However, extraction efficiency of normorphine was between 60 and 70% at levels of 20, 80 and 400 ng/ml, with coefficients of variation between 6 and 10%. Within-run imprecision at these levels was typically <6% for morphine, morphine-3-glucuronide and morphine-6-glucuronide, but higher for normorphine. Between-run imprecision at these levels was typically <10% for morphine, morphine-3-glucuronide and morphine-6-glucuronide, but, again, was higher for normorphine.

TABLE I

REPRODUCIBILITY (COEFFICIENTS OF VARIATION) OF DIFFERENT EXTRACTION DEVICES

Method	*n*	Coefficient of variation (%)			
		Morphine-3-glucuronide (500 ng/ml)	Morphine-6-glucuronide (50 ng/ml)	Normorphine (50 ng/ml)	Morphine (50 ng/ml)
Individually controlled multiple extraction using device described	11	4.5	6.2	9.6	5.2
Multiple extraction	11	6.5	9.6	9.2	8.6
Individual extraction	8	6.1	5.0	8.1	7.7

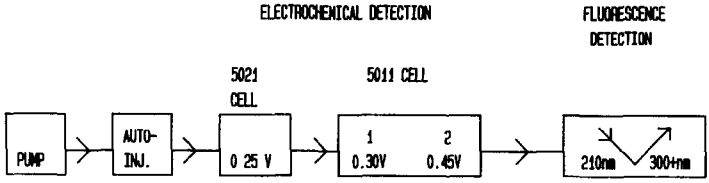

Fig. 2. Detector arrangement for the HPLC method described.

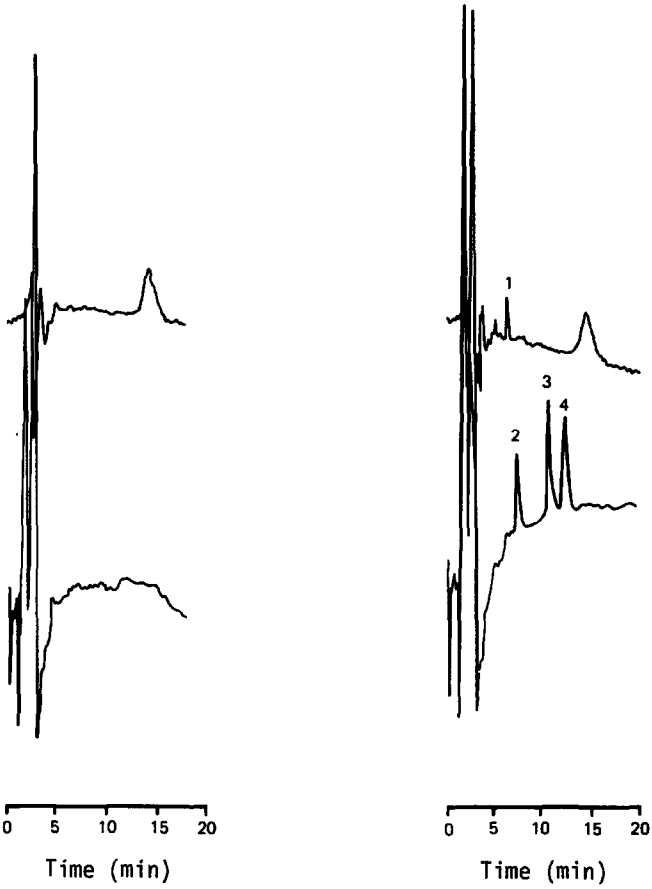

Fig. 3. (Left) Chromatograms of extracted blank plasma showing fluorescence detection (top trace) and electrochemical detection (lower trace). (Right) Chromatograms of extracted plasma spiked with morphine-3-glucuronide (1), 50 ng/ml (top trace, fluorescence detection) and morphine-6-glucuronide (2), normorphine (3) and morphine (4), all 10 ng/ml (lower trace, electrochemical detection).

Chromatography

Electrochemical response to morphine, morphine-6-glucuronide and normorphine was found to vary both between cells and in the same cell with use. For analytical purposes the cell oxidation potentials were typically set at +0.30 V for the first (screening) electrode, and at +0.45 V for the second (analytical) electrode, although this was optimised for different cells.

Analysis of the current–voltage response for a peak found in blank plasma which interferes with the detection of morphine showed a plateau indicating full oxidation of the electrochemically active group at a potential of +0.30 V. The screening electrode, although operating at 0.30 V, was found not to oxidise all of this compound (efficiency approximately 95%), and a further electrode was therefore placed before the main electrochemical cell and operated at a potential of +0.25 V as shown in Fig. 2. This permitted complete removal of the interfering peak. Chromatograms of extracted blank plasma and plasma spiked with morphine-3-glucuronide, morphine-6-glucuronide, normorphine and morphine are shown in Fig. 3.

Limits of detection for this method are 1 ng/ml for morphine-6-glucuronide, morphine and normorphine, and 5 ng/ml for morphine-3-glucuronide.

DISCUSSION

A sensitive and specific method for the determination of morphine and its metabolites in body fluids with rapid sample preparation is reported.

The automated extraction device described permits preparation of multiple samples with flow control through individual cartridges. This device standardises sample extraction without compromising reproducibility, and by control of fluid flow prevents the passage of air through cartridges which can reduce extraction efficiency. The device also overcomes the laborious, time-consuming extraction of single samples, and permits the preparation of at least twice as many samples in the same time period.

An important addition to the method of Svensson et al. [5] is the use of a further screening electrode to fully remove the peak which interferes with the quantitation of morphine. Without this extra electrode a peak equivalent to 5–10 ng/ml morphine is often detected at the analytical electrode in extracted blank plasma, thus limiting ultimate sensitivity of the method for morphine. When the interfering peak is fully removed, quantitation of morphine, normorphine and morphine-6-glucuronide down to levels of 1 ng/ml is possible.

The electrochemical response was found to vary both between electrochemical cells and within the same cell with time. It is therefore necessary to re-evaluate the response of cells periodically during prolonged use and to assess individual cells prior to use. Final potential settings for the analytical cell will inevitably be a compromise between method sensitivity and the detection of other interfering compounds.

The use of morphine-6-glucuronide as a reference standard in this method enables morphine-6-glucuronide to be measured both more reliably and more reproducibly as, although the electro-active group on this compound is the same as that of morphine, their electrochemical response at a particular potential is not identical.

The use of fluorescence detection allows the quantitation of morphine-3-glucuronide down to levels of 5 ng/ml and is a more specific method of detection, especially at low wavelengths (210 nm), than the UV detection previously described.

In conclusion, the modifications to the method of Svensson et al. [5] described here render the technique more rapid and more specific, and the use of a screening electrode ensures sensitivity down to 1 ng/ml with electrochemical detection.

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