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Determination of morphine-3-glucuronide in human urine by capillary zone electrophoresis and micellar electrokinetic capillary chromatography

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ABSTRACT

Attempts to determine morphine-3-glucuronide (MO3G) by high-performance capillary electrophoresis and micellar electrokinetic capillary chromatography are reported. Using direct injection of urine, it was possible to achieve a limit of detection of about 20 μg/ml, which is poor compared with high-performance liquid chromatography and immunoassays. However, employing sample extraction with C_8 cartridges, the presence of MO3G in urines that tested positive for opioids using a commercial enzyme-multiplied immunoassay technique could be successfully confirmed. The limit of detection with unambiguous identification of MO3G via spectral analysis was about 1 μg/ml.

INTRODUCTION

The determination of opioids in biological samples has relevant applications in therapeutic drug monitoring, pharmacodynamic, pharmacokinetic and pharmacogenetic research, as well as clinical and forensic toxicology. Opioids, including heroin and codeine, are metabolized to morphine, and morphine to morphine-3-glucuronide (MO3G) and various other pharmacologically active compounds [1–5]. Thus, there is a clear need for a sensitive and specific method for the determination of free and glucuronidated opioids [6]. The importance of the determination of glucuronidated opioids in body fluids is further underlined in several recent chromatographic publications [7–12].

Recently, micellar electrokinetic capillary chromatography (MECC) was found to be an attractive approach for the analysis of urinary barbiturates [13], drugs of abuse and/or their metabolites, including unconjugated opioids, benzoylecgonine, amphetamines and methaqualone in human urine [14,15], 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid in human urine [16] and a wide range of illicit substances in seizure samples [17]. Furthermore, the determination of cocaine and morphine in human hair by capillary zone electrophoresis (CZE) has been reported [18]. The objectives of the work described in this paper were (i) to determine MO3G in human urine by CZE and MECC with direct sample injection, (ii) to investigate the performance of dif-

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ferent bonded phases for sample clean-up prior to CZE and MECC and (iii) to use these methods to confirm the presence of MO3G in urine samples that tested positive for opioids using an enzyme-multiplied immunoassay technique (EMIT), an assay that is widely employed for screening purposes in forensic and clinical toxicology laboratories.

EXPERIMENTAL

Chemicals, origin of samples and drug screening

All chemicals used were of analytical or research grade. MO3G, prepared according to Yoshimura *et al.* [19] or purchased from Sigma (St. Louis, MO, USA), served as standard compound. Urine samples were collected in our routine drug assay laboratory where they were received for drug screening. A sample containing MO3G was also obtained by self-administration of twenty drops of a cough medicine containing codeine (about 5 mg) (Resyl Plus, Ciba-Geigy, OTC Pharma, Basle, Switzerland) and urine collection after 5 h. Our own urine was employed as blank matrix. The samples were screened for the presence of opiates using an automated enzyme-multiplied immunoassay technique (EMIT-dau, Syva, Palo Alto, CA, USA) on a Cobas Fara centrifugal analyser (Hoffmann-La Roche, Diagnostica, Basle, Switzerland). In this assay, morphine was employed as reference substance. Samples that gave a response equal or higher than 300 ng/ml were interpreted as positive.

Electrophoretic instrumentation

The instrument with multi-wavelength detection employed in this work was described previously [13–16]. Briefly, it featured a 75 μm I.D. fused-silica capillary of about 90 cm length (Product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA) together with a Model UVIS 206 PHD fast-scanning multi-wavelength detector with No. 9550-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA) placed towards the capillary end. The effective separation distance was 70 cm. A constant voltage of 20 kV was applied using an HCN 14-20000 power supply (FUG Elektronik, Rosenheim, Germany). The cathode was on the detector side. For sample application the anodic capillary end was dipped into the sample vial and lifted some 34 cm for about 5 s. Multi-wavelength data were read, evaluated and stored employing a Mandax AT 286 computer system and running the 206 detector software package version 2.0 (Linear Instruments) with windows 286 version 2.1 (Microsoft, Redmont, WA, USA). Throughout this work the 206 detector was employed in the high-speed polychrome mode by scanning from 195 to 210 nm at 5-nm intervals (26 wavelengths). Conditioning for each experiment occurred by rinsing the capillary with 0.1 *M* sodium hydroxide for 3 min and with buffer for 5 min.

Buffer system

For MECC, a buffer composed of 75 m*M* sodium dodecyl sulphate (SDS), 6 m*M* sodium tetraborate and 10 m*M* disodium hydrogenphosphate (pH about 9.2) was employed. In some cases, this buffer was modified with acetonitrile (10%, v/v) and/or with 1 *M* sodium hydroxide to increase the pH. For CZE, a buffer composed of 12 m*M* sodium tetraborate and 20 m*M* disodium hydrogenphosphate was used and adjusted to the desired pH (range 8.2–10.2) by addition of sodium hydroxide or hydrochloric acid (about 1 *M* each).

Sample pretreatment

Disposable solid-phase cartridges, Bond-Elut C_2, C_8, C_{18}, SCX and Certify (Analytichem International, Harbor City, CA, USA), as well as Bakerbond spe C_{18} (40 μm, 60 Å, J. T. Baker, Phillipsburg, NJ, USA), were used. For all types of columns the Vac Elut set-up (Analytichem International) served as cartridge holder and vacuum manifold. Extended drying periods of the sobents were avoided. The extraction procedure employed was similar to those reported by Svensson *et al.* [6] and Glare *et al.* [9]. The cartridges were conditioned immediately prior to use by passing sequentially 5 ml of methanol, 3 ml of 10 m*M* potassium dihydrogenphosphate containing 10% acetonitrile adjusted to pH 2.1 by addition

of phosphoric acid, and 5 ml of distilled water. The vacuum was turned off as soon as the applied solutions reached the sorbent bed to prevent column drying. A 10-ml aliquot of a 0.5 *M* ammonium sulphate–ammonia buffer (pH 9.3) was added to 5 ml of urine and this mixture was applied and slowly aspirated (1–2 ml/min) through the cartridge. The column was sequentially rinsed with 10 ml of a 5 m*M* ammonium sulphate–ammonia (pH 9.3) buffer and 0.5 ml of distilled water. The vacuum [about 1.33 kPa (10 mmHg) for 15–20 s] was turned off as soon as the last drop of the aqueous solution fully penetrated the column. Elution occurred by slowly (about 1 ml/min) aspirating 2 ml of 10 m*M* potassium dihydrogenphosphate containing 10% acetonitrile, which was adjusted to pH 2.1 by addition of phosphoric acid. Then the eluate was frozen (liquid nitrogen) and freeze-dried prior to reconstitution in 100 μl of water. As an alternative, the eluate could be dried within a few hours at 40°C and employing a gentle stream of nitrogen. In a second elution step, applied for the measurement of the amount of MO3G left within the column, 2 ml of methanol were slowly passed through the column, the eluate was evaporated to dryness under a gentle stream of nitrogen at room temperature and the residue was dissolved in 100 μl of water.

Recovery

The recovery after sample pretreatment was determined by comparing CZE or MECC peak heights after extraction with peak heights obtained by direct injection of equal amounts of MO3G dissolved in water.

RESULTS AND DISCUSSION

Opioids and other illicit drug substances have been shown to be well separated using a phosphate–tetraborate buffer containing 75 m*M* SDS (pH 9.2) [14–16]. Fig. 1 depicts the three-dimensional electropherogram of MO3G (Fig. 1A) as well as the normalized absorption spectrum (Fig. 1B) obtained from the gathered data. Under these experimental conditions MO3G was found to reach the detector in about 9 min, an elution time that is significantly shorter than that of benzoylecgonine and other opioids, including morphine, 6-acetylmorphine, codeine and heroin [14,15], and similar to that of caffeine [20].

For MECC with on-column UV absorption detection the typical sensitivity is of the order of μg/ml (μ*M*), a concentration level that is common for urinary MO3G. Thus the feasibility of direct urine injection was investigated. The electropherogram depicted in Fig. 2A was obtained by direct application of a urine blank spiked with 50 μg/ml MO3G and using the same buffer as for Fig. 1. A rather complex electropherogram was yielded with MO3G coeluting with one or several endogenous compound(s). Addition of acetonitrile (10%, v/v; Fig. 2B) did spread the pattern, but without providing an interference-free elution of MO3G. Similar results were obtained in the CZE mode, *i.e.* without the use of SDS (Fig. 2C). In all these cases, however, MO3G peak identification via comparison of spectra was easily possible down to the 30 μg/ml level (data not shown). Better results in terms of interference-free elution were obtained within CZE at a pH of

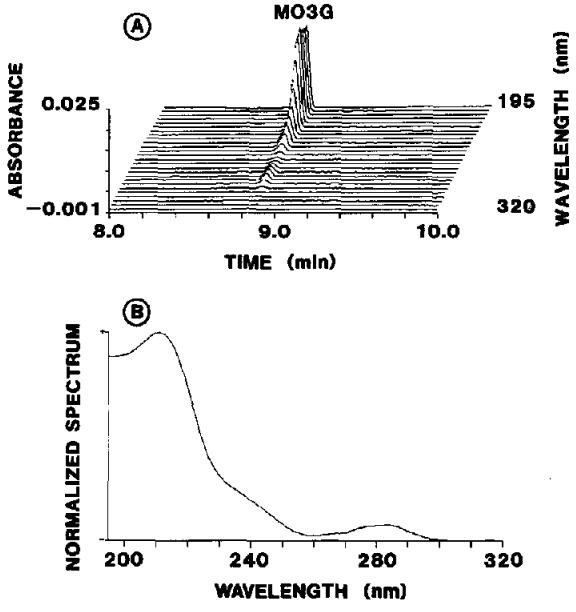

Fig. 1. (A) Multi-wavelength MECC data of MO3G (35 μg/ml). The current was 76 μA. (B) Extracted, normalized absorption spectrum of MO3G.

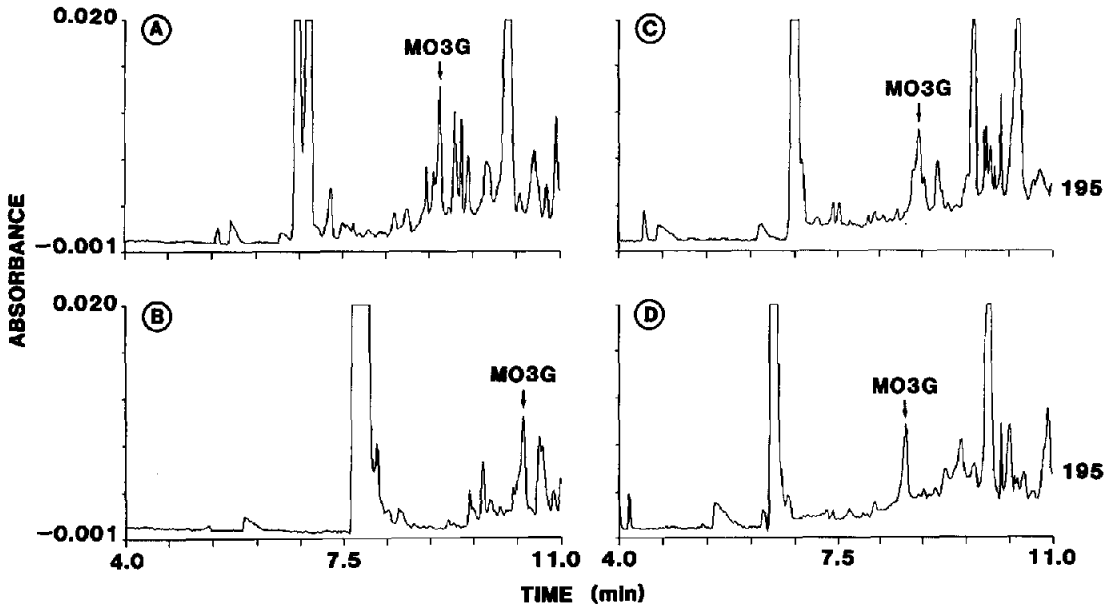

Fig. 2. Single-wavelength (195 nm) electropherograms of a directly injected urine blank spiked with MO3G (50 μg/ml). (A) MECC at pH 9.2; (B) MECC in the presence of 10% acetonitrile at pH 9.2; (C) CZE at pH 9.2; (D) CZE at pH 9.8. The currents were 72, 77, 80 and 100 μA, respectively.

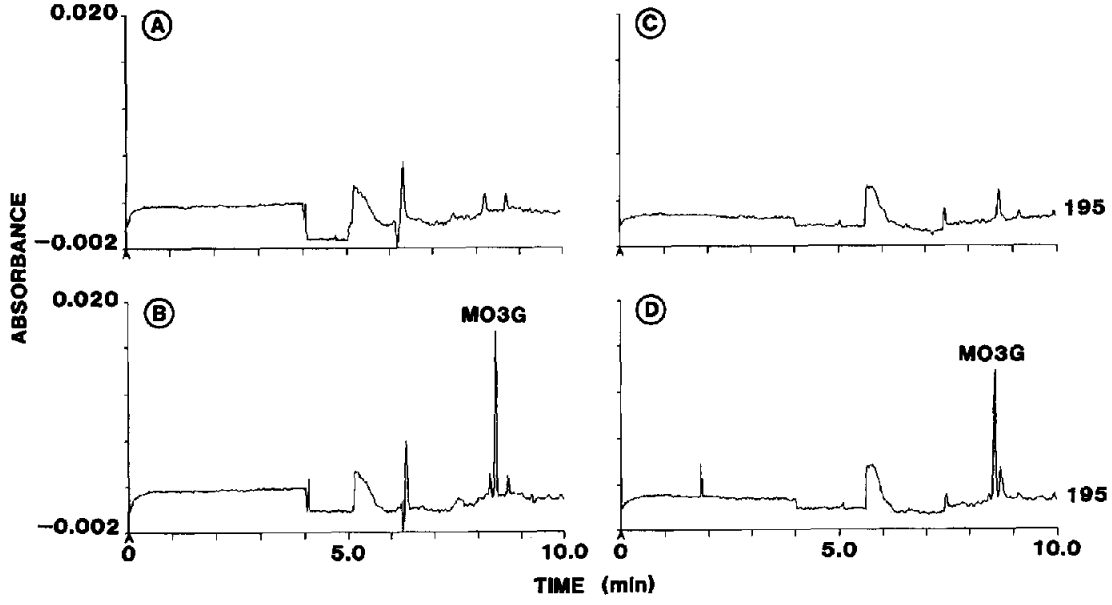

Fig. 3. CZE (A and B) and MECC (C and D) electropherograms obtained after extraction with C_8 cartridges of (A and C) urine blank and (B and D) urine blank spiked with 10 μg/ml MO3G. The buffer pH in all measurements was 9.8. The currents for CZE and MECC runs were 102 and 80 μA, respectively.

9.8 (Fig. 2D), achieving a limit of unambiguous identification of 20 μg/ml. Unfortunately, this is still too high for our purposes. In fact, urines that tested EMIT-positive for opioids did not provide a detectable MO3G signal with direct urine injection. Thus, MO3G extraction appeared to be essential.

Using a C_{18} extraction procedure derived from those of Svensson *et al.* [6] and Glare *et al.* [9], MO3G recoveries were not better than about 20% employing urine blank spiked with 10 μg/ml MO3G. Some (but not all) of the missing glucuronide could be recovered in a subsequent elution with methanol. Similar results were obtained employing C_{18} cartridges from two different manufactures (see Experimental section). Unfortunately, using methanol as eluent resulted in an increased recovery not only of MO3G, but also of interfering matrix compounds, which offset any improvement in sensitivity. Thus, insufficient selectivity for MO3G during elution from the cartridges appeared to be the key problem in this cleaning up procedure. Preconditioning the columns with an ammonium acetate solution (0.3 *M*, pH about 8), applying methylene chloride as eluent, changing the pH of the wash solution after sample application, and repeating the extraction (as originally suggested by Svensson *et al.* [6] did not appreciably improve results.

A significant improvement in recovery (about 60%) and cleanness of the extracts was achieved with C_8 columns. CZE and MECC electropherograms of a urine blank and a urine blank spiked with 10 μg/ml MO3G are presented in Fig. 3. In all cases, the reconstituted extract was ten-fold diluted prior to analysis. Both CZE and MECC at pH 9.8 appear to be suitable for the determination of MO3G, with CZE being somewhat less prone to matrix interferences. Using 5 ml of urine, the detection limit for unambiguous identification via spectral analysis was determined to be about 1 μg/ml, this being about a twenty-fold improvement compared with the data with direct urine injection. On the basis of these data, the C_8 extraction procedure was used for MO3G determination in patient urines preliminarily screened by EMIT. CZE electropherograms of two urine specimens that were found to be markedly positive for opiates using EMIT are depicted in Fig. 4. In both the urine specimen collected after self-administration of codeine (Fig. 4A) and the patient urine (Fig. 4B), the presence of MO3G could easily be confirmed. The excellent agreement between the normalized time slice (absorption spectrum) of the MO3G peak of Fig. 4B with that of a computer-stored standard is documented with the graphs shown in Fig. 4C. With a simple calibration graph based on peak heights, the MO3G concentrations were estimated to be around 4 and 2.5 μg/ml for the urines of Fig. 4A and B, respectively.

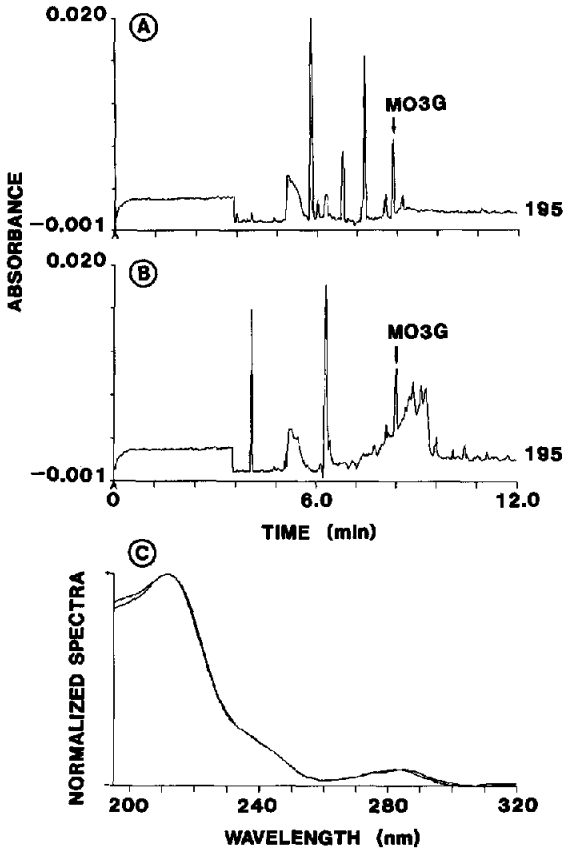

Fig. 4. CZE electropherograms obtained after extraction with C_8 cartridges of (A) urine collected after self-administration of a cough medicine containing codeine and (B) a patient sample. The buffer pH was 9.8 and reconstituted samples were ten-fold diluted prior to analysis. C depicts the spectral identity proof comprising background subtracted, normalized spectra of the MO3G peaks of B and Fig. 3B. Other conditions are the same as those of Fig. 3B.

Modifying the extraction procedure by using eluents with either an increased acetonitrile content (up to 50%) and/or pH (up to 5) provided increased recovery of MO3G as well as matrix interference, thus not providing any improvements in comparison with the method described above. Also, no gain was observed by lowering the pH (down to pH 5) in the sample application step and in the subsequent wash. Furthermore, no improvements were noted employing C_2 instead of C_8 columns, while the Bond-Elut Certify "mixed-mode" columns used previously for drug confirmation testing [14,15] were found to be useless for MO3G extraction.

All extractions described above include time-consuming freeze-drying (or evaporation under a stream of nitrogen) prior to reconstitution with a small amount of water. Recently, Chari *et al.* [10] described the use of SCX (strong cation exchange resin) cartridges for extraction of MO3G and other opioids followed by their determination with HPLC. In this method, urine is applied at low pH (about 2.5) and methanol containing 3% ammonia (pH about 11) is employed as eluent. This procedure would be very appealing because it would permit rapid drying of extracts, but could not be reproduced for MO3G in our laboratory. After application of a standard solution (without the urine matrix) or blank urine spiked with MO3G (both containing 10 μg/ml MO3G), MO3G could be found neither in the drawn sample solution nor in the wash solution, and not at all in the eluate. Thus, MO3G is assumed to remain completely adsorbed onto the resin.

In conclusion, the data described in this paper reveal that MO3G in urine can be analysed by MECC and CZE using pH 9.8 buffers and sample extraction with C_8 cartridges. The presence of MO3G could be demonstrated in our own urine, which was collected after self-administration of a codeine-containing coughing medicine, as well as in patient urines.

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