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Determination of methadone and its primary metabolite in human urine by capillary electrophoretic techniques

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Abstract

Data presented in this paper show that methadone and its primary urinary metabolite (M_1) can not easily be determined by SDS-based micellar electrokinetic chromatography; however, they separate rapidly under cationic capillary zone electrophoretic conditions using a borate buffer with a pH of ca. 9. Eight urines obtained from individuals undergoing methadone therapy, which tested markedly positive for methadone using an enzyme multiplied immunoassay and in which the presence of methadone and M_1 was also confirmed by GC–MS, have been analyzed. Using an extraction procedure with disposable cartridges containing a copolymeric sorbent, the presence of methadone and M_1 could be confirmed in all urines, whereas with direct urine injection, the two compounds could only be determined in six urines. Thus, for unambiguous confirmation by capillary electrophoresis, extraction of the compounds of interest is preferred. The described assay is rapid (with typical run times being less than 6 min), free of interferences from coextracting drugs of abuse and/or their major metabolites, and characterized by a good reproducibility. After extraction of 5 ml urine, drug concentrations down to ca. 20 ng/ml can be monitored unambiguously.

1. Introduction

Methadone (for structure see Fig. 1) is an analgesic drug with pharmacological properties similar to those of morphine. Primary uses are relief of pain and part of the treatment of dependence from opioid drugs (treatment of heroin addiction combined with efforts at social rehabilitation). Doses of ca. 5–10 mg are commonly used to evoke analgesia, doses between 30 and 150 mg (carefully adjusted for each individual) are given daily during opioid withdrawal treatments. Methadone is absorbed well from the gastrointestinal tract and ca. 90% of the drug is bound to plasma proteins. After extended biotransformation in the liver, the major metabolites and unchanged drug are excreted in the urine and in the bile with an elimination rate of ca. 20–60% of the dose within 24 h [1–5]. Urinary compounds mainly include methadone and its primary metabolite (M_1, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, often referred to as EDDP) which results through N-demethylation and cyclization (for structure see Fig. 1). Moreover, the presence of trace amounts of a secondary metabolite (M_2, 2-ethyl-5-methyl-3,3-diphenylpyrroline, EMDP, Fig. 1) and of a decomposition product of M_1 have been re-

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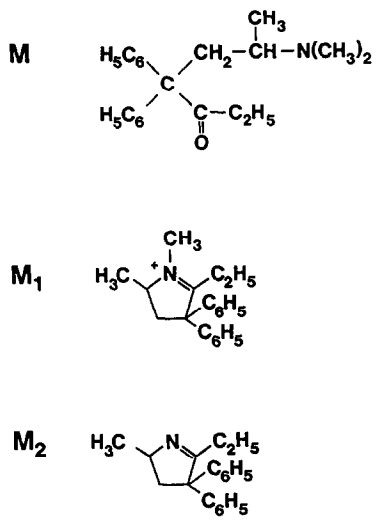

Fig. 1. Chemical structures of methadone (M), its primary urinary metabolite (M_1) and its secondary urinary metabolite (M_2).

ported [6]. It is further known, that the urinary ratio of primary metabolite to methadone is typically <1 for acute treatments and >1 for chronic treatments with methadone, and that the 24-h recovery of methadone and M_1 in urine is increased during chronic administration [5].

Determination of urinary methadone is important to monitor patients undergoing methadone therapy. Because of the possible presence of other drugs in urines of these individuals, methods that allow the simultaneous monitoring of methadone and other common drugs of abuse or approaches permitting a selective determination of methadone in the presence of such compounds (without interferences) are required. Screening patient samples for methadone is typically performed with immunological methods, including enzyme multiplied immunoassay technique (EMIT) and fluorescence polarization immunoassay (FPIA). Other methods employed are gas chromatography [6,7] or gas chromatography–mass spectrometry (GC–MS) [8], high-performance liquid chromatography [9], or voltammetry [10]. With most of these techniques [6,8,9], simultaneous determination of methadone and M_1 has been reported, the analysis of the latter compound being important for the investigation of compliance.

Recently, the feasibility of monitoring illicit drugs and/or their metabolites (including opioids, amphetamines, cocaine metabolites, cannabinoids, barbiturates, benzodiazepines and methaqualone) in human urine by micellar electrokinetic capillary chromatography (MECC) with on-column fast scanning polychrome absorption detection has been reported [11–14]. The objectives of the work described in this paper were (i) to investigate different electrokinetic capillary approaches [based upon MECC and capillary zone electrophoresis (CZE)] for the determination of urinary methadone and M_1, (ii) to evaluate different sample preparation methods, including direct urine injection and solid-phase extraction procedures, and (iii) to use capillary electrophoresis with on-column multiwavelength absorption detection to confirm the presence of methadone and M_1 in urine samples which tested positive for methadone using both EMIT and GC–MS.

2. Experimental

2.1. Chemicals

All chemicals used were of analytical or research grade. Reference substances, including methadone, were of European Pharmacopoeia quality. dl-Methadone primary metabolite (HI salt) and dl-methadone secondary metabolite (HCl salt) were purchased from Alltech-Applied Science Labs (State College, PA, USA). Benzodiazepine metabolites (3-hydroxybromazepam, 7-aminoclonazepam, desmethyldiazepam, 7-aminoflunitrazepam and α-hydroxymidazolam) were a kind gift of Hoffmann-La Roche (Basel, Switzerland).

2.2. Origin of samples and immunological drug screening

Urine samples were collected at the Institute of Pharmacy where they were received for regular control of patients undergoing methadone

therapy (daily dosage of ca. 60 mg). These urines are typically analyzed for methadone and drugs of abuse using immunological screening methods and GC–MS. For the purpose of the work described here, methadone and its primary metabolite were determined in 8 urines by the latter technique (see below). Furthermore, in the drug assay laboratory of the Department of Clinical Pharmacology, the urines were screened for the presence of methadone using an enzyme multiplied immunoassay technique (EMIT, EMIT d.a.u., Syva, Palo Alto, CA, USA) on a Cobas Fara centrifugal analyzer (Hoffmann-La Roche, Diagnostica, Basel, Switzerland). The EMIT test contains methadone as calibrator with a cut-off level of 300 ng/ml and a positive control of 1000 ng/ml. Samples which gave an equal or higher response than the cut-off calibrator were interpreted as positive and samples which gave a higher response than the mean of the cut-off and control calibrators (>650 ng/ml) were interpreted as markedly positive. Our own urine was used as blank urine. The samples were stored at −20°C until further analysis.

2.3. GC–MS of urinary methadone and its primary metabolite

Liquid–liquid extraction was performed without hydrolysis according to the following procedure. A 6-ml volume of ethyl acetate was added to a mixture of 20 ml of urine and 10 g of $(NH_4)_2SO_4$ which was adjusted to pH >8.5 with ca. 1.5 ml of concentrated NH_3 (25%), and extraction was performed in a separation funnel. After vigorous shaking for 1 min, the organic phase was centrifuged at ca. 1500 *g* for 5 min prior to acidification with 3 ml of an aqueous 10% (w/v) solution of tartaric acid. After shaking for 2 min, phase separation was executed and the organic phase was discarded. The drug was reextracted by addition of 2 g of $(NH_4)_2SO_4$ to the aqueous phase, adjustment to pH >8.5 with ammonia (see above), and by use of 400 μl of ethyl acetate. Aliquots of 1 μl of the organic phase were injected onto a Model HP 5890 GC (Hewlett-Packard, Widen, Switzerland) equipped with a temperature programmer and a Model HP 7673A autosampler (Hewlett-Packard). A DB-5 column (20 m × 0.18 mm I.D. 0.4 μm film thickness; J&W, Folsom, CA, USA) was used together with a splitless injector which included a liner (RDL-1076-H-22ga, chambered split/splitless; R&D, Rancho Cordova, CA, USA). The temperatures of the injector and interface were 260 and 280°C, respectively. Initial and final column temperatures were 70 and 290°C, respectively. The temperature was increased by ramping first from $t = 1.0$ min to $t = 9.67$ min (70 to 200°C) at a rate of 15°C/min and then from $t = 9.67$ min to $t = 14.67$ min (200 to 290°C) at a rate of 18°C/min. The total run time was 24 min. The MS detector was a Model HP 5970 MSD (Hewlett-Packard) operating in the scan acquisition mode between 33 and 400 amu. An HP 59970 MS ChemStation (version 3.2, Hewlett-Packard) was employed as data station and for data evaluation. Data acquisition was initiated 4 min after sample injection.

2.4. Electrophoretic instrumentation and running conditions

Two instruments with multi-wavelength absorption detection were employed. If not stated otherwise, a 50 m*M* tetraborate buffer ($Na_2B_4O_7$) of pH about 9.3 (without any additives) was used as running buffer in both apparatuses. The laboratory made setup [11–13] featured a ca. 90 cm × 75 μm I.D. fused-silica capillary (product TSP/075/375, Polymicro Technologies, Phoenix, AZ, USA) together with a Model UVIS 206 PHD fast-scanning multiwavelength detector with a No. 9559-0155 on-column capillary detector cell (both from Linear Instruments, Reno, NV, USA) towards the capillary end. The effective separation distance was 70 cm. A constant voltage of 20 kV (current: ca. 90 μA) was applied. Throughout this work the detector was employed in the high-speed polychrome mode by scanning from 195 to 320 nm at 5 nm intervals. Before each experiment the capillary was rinsed with 0.1 *M* NaOH and running buffer for 5 min each. The second instrument used was the fully automated BioFocus 3000 capillary electrophoresis system

(Bio-Rad Laboratories, Hercules, CA, USA). It was equipped with a 50 μm I.D. fused-silica capillary of 50 cm total length (45 cm to the detector) mounted in a user assembled cartridge (Bio-Rad). Injection of sample was effected by applying a pressure of 34.45 kPa s (5 psi s). A constant voltage of 15 kV (current: 39 μA) was applied, the temperature of the cartridge was maintained at 20°C and detection was effected at 195 nm. For identification purposes, the fast-scanning detection mode (range: 195 and 320 nm at 5 nm intervals) was employed. BioFocus and Spectra software (version 3.00, Bio-Rad) was used for the control of the instrument, the execution of the runs, for data acquisition and storage, and qualitative evaluation of multiwavelength data. BioFocus Integration software (version 3.01, Bio-Rad) was employed for data conversion and evaluation on the basis of peak areas. Capillary equilibration between runs was obtained by rinsing the capillary with 0.1 *M* NaOH (40 s) and with buffer (100 s) employing the high pressure mode. After each set of 10 runs, a new anodic buffer vial of 5 ml volume was used, this ensuring constant running conditions.

2.5. Sample pretreatment for electrokinetic capillary analyses

Standard solutions of methadone and its metabolites were prepared in methanol at a concentration of ca. 2 mg/ml. For analysis the standard solutions were diluted with running buffer or water. Blank urine was spiked with known aliquots of standard solutions to the urine. Urine samples were injected as received or, prior to analysis, drugs were extracted using Bond Elut Certify cartridges and a Vac Elut setup (both from Analytichem International, Harbor City, CA). Two different procedures were evaluated. First, the two-step method described before [12] was used. The cartridges were conditioned immediately prior to use by passing sequentially 2 ml of methanol and an equal volume of 0.1 *M* phosphate buffer (pH 6) through the columns. The vacuum was turned off to prevent column drying. The columns were loaded by slowly (about 2 min) drawing of a mixture of 5 ml of urine and 2 ml of 0.1 *M* phosphate buffer (adjusted to pH 6). The columns were then rinsed sequentially with 1 ml of 0.1 *M* phosphate buffer–methanol (80:20, v/v), with 1 ml of 1 *M* acetic acid and with 1 ml of hexane. In contrast to the procedure employed by Wernly and Thormann [12], after each rinse the columns were dried under full vacuum as described by the manufacturer for 5, 10 and 2 min, respectively. Full vacuum drying was found to provide a somewhat higher recovery for M_1, no change for methadone, and a smaller system peak. The first elution of drugs occurred with 4 ml of methylene chloride into a clean test tube. The cartridges were then rinsed with 6 ml of methanol. A second elution was achieved with 2 ml methylene chloride–isopropyl alcohol (80:20, v/v) containing 5% (v/v) concentrated ammonium hydroxide solution. As an alternative, the extraction procedure for amphetamine/metamphetamine recommended by the manufacturer of the solid-phase extraction columns was used. Preparation of the urine specimens and conditioning of the cartridges were performed in the same way as described above for the two-step extraction. After sample application, the columns were rinsed with 1 ml of 1.0 *M* acetic acid and 6 ml of methanol and dried under full vacuum for 5 and 2 min, respectively. Elution occurred with 2 ml of ethyl acetate containing 2% (v/v) concentrated ammonium hydroxide solution. Independent of the extraction procedure applied, the eluates were evaporated to dryness under a gentle stream of air at room temperature and the residues were dissolved in 50 μl buffer, diluted running buffer or water.

3. Results and discussion

3.1. Separation of methadone and M_1 by MECC

The data presented in Fig. 2 illustrate the different steps in the development of a method for the determination of methadone and its metabolites in urine. As previously described,

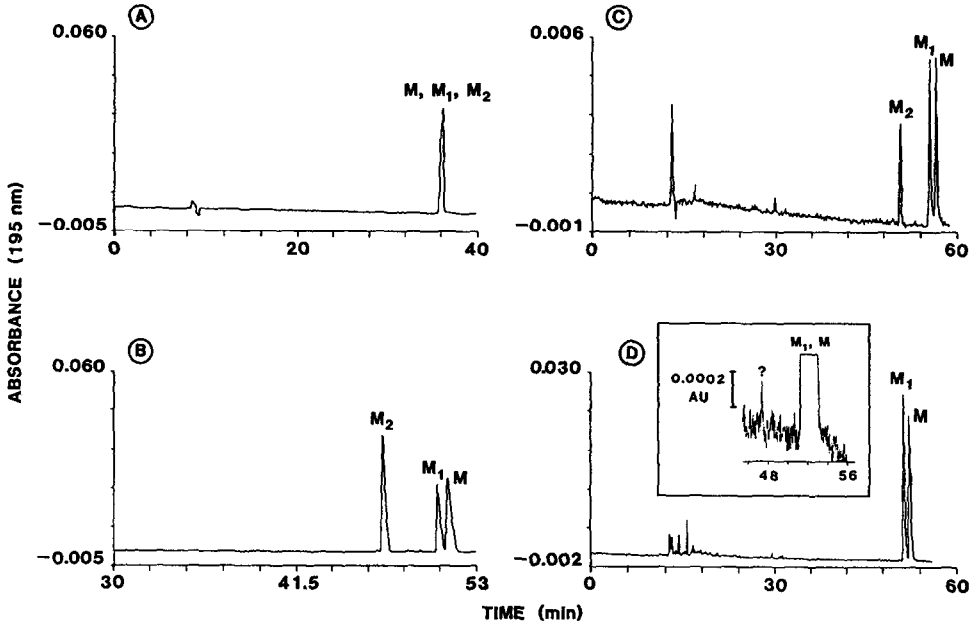

Fig. 2. MECC electropherograms of (A) methadone and the two metabolites (ca. 100 μg/ml each) using a buffer composed of SDS 75 m*M*, phosphate 10 m*M* and tetraborate 6 m*M* (pH 9.2), (B) a similar sample as in A but with a buffer containing SDS 12.5 m*M*, phosphate 10 m*M*, tetraborate 6 m*M* and isopropyl alcohol 8% (v/v) (pH 9.4), (C) an extract from a blank urine spiked with the three compounds (ca. 10 μg/ml each) and analyzed with the buffer used to produce the data of panel B, and (D) the same as for panel C but with an extract of a patient urine (urine No. 1). The laboratory-made instrument was employed. A constant voltage of 20 kV was applied in all cases and the currents were 57 μA (panel A) and 27 μA (panels B–D). Key as for Fig. 1.

methadone could not be determined by MECC with the buffer generally used for the analysis of different drugs of abuse [11,12]. In this system composed of 75 m*M* SDS, 10 m*M* Na_2HPO_4 and 6 m*M* $Na_2B_4O_7$ (pH 9.2–9.3), methadone and its two metabolites were found to completely partition into the micelles and to emerge as one peak (Fig. 2A). Different parameters were investigated in order to separate the three substances (Fig. 1). No resolution was observed after variation of the buffer pH in the range between 8.5 and 10.0 (data not shown). With addition of isopropyl alcohol (5% v/v, as in Ref. [14]), M_2 was found to emerge in front of the coeluting compounds methadone and M_1, but complete separation of the compounds was not observed. On the other hand, with decreasing SDS concentration, the secondary metabolite could be completely separated from the other two components. However, complete separation of all three compounds could only be achieved employing both a low SDS concentration and an organic buffer modifier. An electropherogram of the standard substances using the modified SDS buffer [SDS 12.5 m*M*, $NaHPO_4$ 10 m*M*, $Na_2B_4O_7$ 6 m*M* and 8% (v/v) isopropyl alcohol] is shown in Fig. 2B. The data presented in panel C of Fig. 2 were obtained with an extract from a blank urine spiked with all three compounds (ca. 10 μg/ml each). With the two-step extraction procedure, methadone and its metabolites were found to elute in the second step only, i.e. employing the methylene chloride–isopropyl alcohol fraction containing 5% ammonium hydroxide solution. The recovery was determined to be about 95%. Analysis of a patient sample after application of the same solid-phase extraction revealed the presence of methadone and its

primary metabolite M_1 (Fig. 2D), whereas the secondary metabolite could not be detected (insert in Fig. 2D; peak marked with ? has equal elution characteristics as M_2 but is too small to be identified). The concentration of the latter compound is too low to be monitored by MECC with UV absorption detection. The SDS concentration used to obtain the data presented in Figs. 2B–D was low (slightly above the critical micelle concentration), but still defined elution order according to differences in partitioning. Furthermore, because of the rather low ionic strength of the buffer, peak distortion was evident (Fig. 2B) and run times were rather high and therefore not suitable for rapid confirmation purposes. With increased ionic strength [buffer composed of 12 m*M* SDS, 30 m*M* tetraborate, 50 m*M* phosphate and 8% (v/v) isopropanol], the same analysis could be performed in a very short time and, interestingly enough, CZE appeared to become dominant and a change in elution order was observed (data not shown, see below). Thus, no further work was done on the elucidation of a more effective MECC approach for determination of methadone and its metabolites.

3.2. *Analysis of methadone and M_1 by CZE with direct urine injection*

CZE was immediately found to be more suited for determination of methadone in urine. Methadone and its two metabolites were separated in a 50 m*M* tetraborate buffer (pH 9.3) in less than 10 min (Fig. 3A). Methadone and M_1 are shown to appear as cations in front of the electroosmotic system peak (marked with S), whereas M_2 and S are shown to be inseparable. Variation of the pH (in the pH range 8.5–10.0), the buffer concentration and composition (phosphate/tetraborate instead of tetraborate) did not greatly affect the separation of methadone and its metabolites. However, in the presence of interfering compounds, these parameters should be exploited for optimization of the separation (see below). Injection of urine blank revealed that most of the (negatively charged) urinary matrix components are transported more slowly

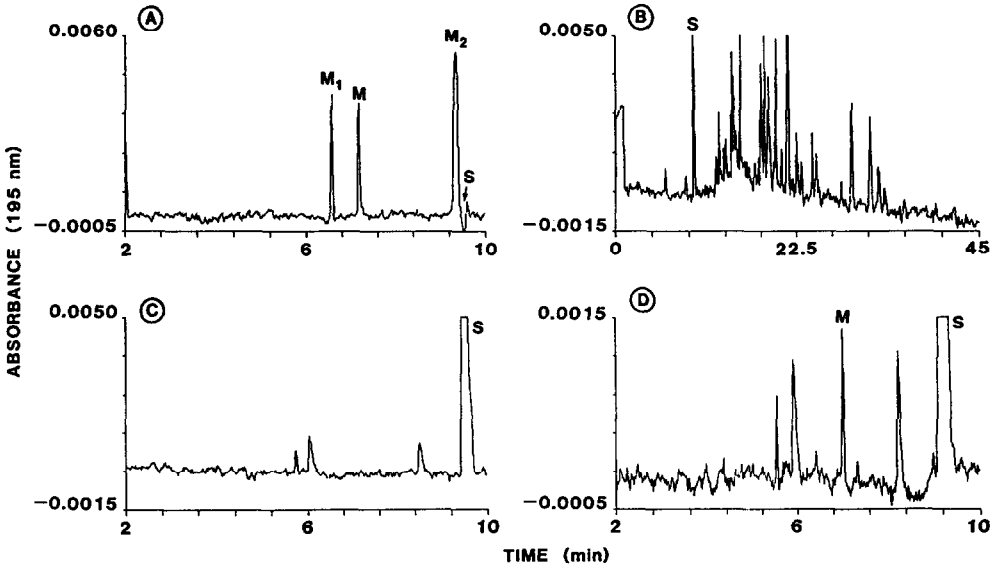

Fig. 3. CZE electropherograms obtained on the laboratory-made setup with (A) the three model compounds (ca. 10 μg/ml each), (B, C) a directly injected urine blank, and (D) a directly injected urine blank spiked with 5 μg/ml methadone. S marks the electroosmotic system peak and the key is the same as for Fig. 1. All other conditions as described in the Experimental section.

than the electroosmotic flow (expressed as peak S) and are therefore detected after 10 min (Fig. 3B). As is shown from the data drawn on an expanded time scale (Fig. 3C), very few minor peaks are detected in front of the electroosmotic flow (between 2 and ca. 9 min, Fig. 3C). This allows the determination of positively charged species in this region, such as methadone, by direct injection of urine (Fig. 3D). Under the conditions tested, M_2 was always migrating with the electroosmotic flow and thus together with other components of the urine matrix (Fig. 3B–D). As this metabolite is not expected to be detected by UV absorbance (see above), attention was focused on only monitoring methadone and M_1. Comparison of the data presented in Fig. 3 revealed that endogeneous compounds present in untreated urine blank should not interfere with methadone and M_1. Indeed, the two substances could be unambiguously monitored by injection of untreated urine in 6 out of 8 patient samples which were found to be positive for methadone by both GC–MS and EMIT. An example is given with the CZE data presented in Fig. 4A, data which were obtained with a markedly EMIT positive urine specimen. Using calibration graphs based on peak heights [linear, four-point calibration with standards of 50, 25, 5 and 1 μg/ml of methadone ($r = 0.996$)], the concentrations of methadone in that patient urine was estimated to be ca. 12 μg/ml. For direct urine injection, the detection limit for unambiguous determination of methadone was found to be ca. 2 μg/ml (between 1 and 5

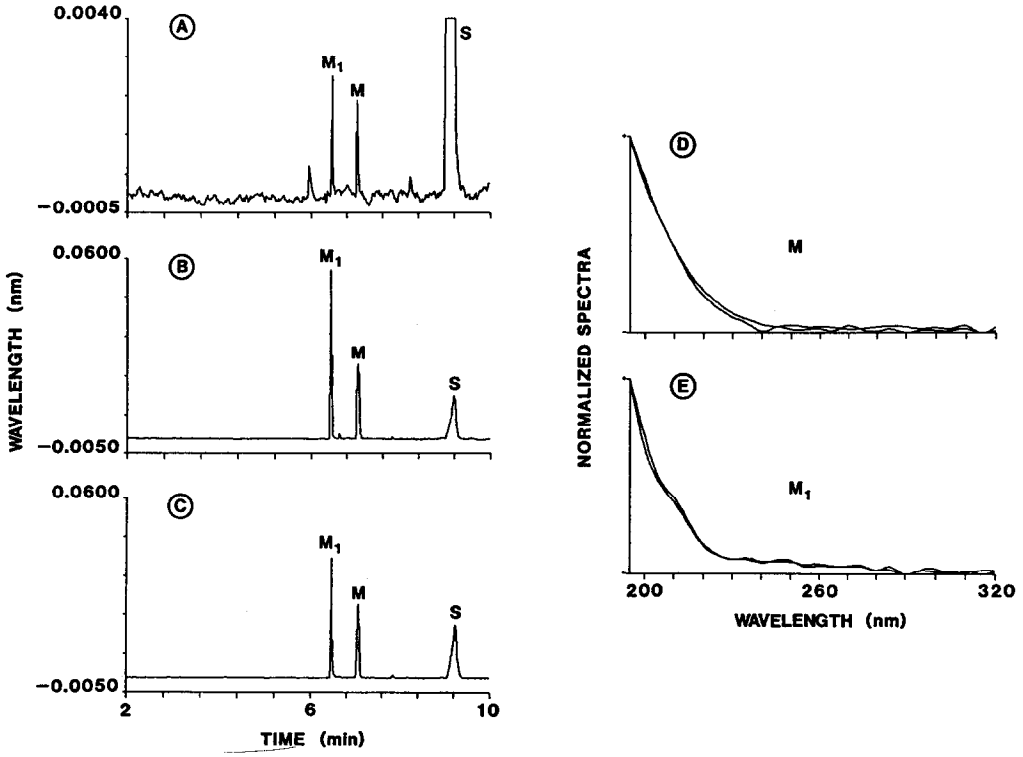

Fig. 4. CZE data of patient urine No. 3 monitored on the homemade apparatus. Electropherograms obtained at 195 nm after (A) direct urine injection, (B) solid-phase extraction using the two-step method (second fraction), and (C) solid-phase extraction using the procedure for amphetamines are depicted. Identity proofs for methadone and M_1 of electropherogram A via comparison of the normalized absorption spectra with those obtained with reference compounds are shown in panels D and E, respectively. Other conditions as for Fig. 3.

μg/ml, this depending on differences in background noise of the investigated urines). Similar data were obtained for M_1.

3.3. Analysis of methadone and M_1 by CZE after extraction

In a second approach, the effects of extraction were investigated. With fraction 2 of the two-step extraction procedure, the detection limit for methadone was found to be between 20 and 50 ng/ml (with 100 fold concentration), this limit being ca. 100 fold lower than that observed with direct urine injection. Comparable data were obtained with the extraction procedure using 2% ammonium hydroxide solution in ethyl acetate as eluent. In both cases, similar electropherograms were obtained (see Figs. 4B and C, respectively). The same urine specimen as used to produce the data of Fig. 4A was employed. It is important to note (i) the absence of any major peaks other than those produced by methadone and M_1, and (ii) the increased peak heights of both compounds compared to those obtained by direct urine injection (panel A). The system peak marking the electroosmotic flow (at ca. 9 min) is obtained in all three cases. For this sample, methadone and M_1 could easily and unambiguously be identified via comparison of normalized absorption spectra with those of the reference substances. Examples for the data of panel A are given in panels D and E.

In two of the eight patient urines analyzed, methadone and M_1 could not be recognized after direct urine injection. These urines were characterized by a high amount of sediment, relative high pH (ca. 8 compared to 5–7 for all other samples) and an unusual, unpleasant odor. After acidification of these urines (pH ca. 6), no change in the electropherogram was observed. An electropherogram of one of the two urines is shown in Fig. 5A. Comparison with Fig. 4A clearly shows the difference between the two sample matrices. If the urines were subjected to solid-phase extraction, however, normal results were obtained with the exception that an additional peak eluting just after methadone was

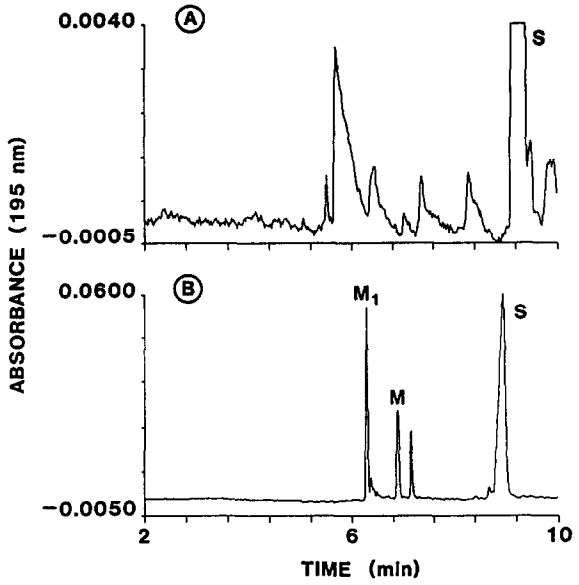

Fig. 5. CZE data of patient urine No. 6 with electropherograms obtained after (A) direct urine injection and (B) sample preparation via two-step solid-phase extraction (second fraction). Other conditions as for Fig. 3.

detected (Fig. 5B). No efforts were undertaken to identify this unknown peak.

3.4. Comparison of data obtained with CZE and GC–MS

Differences in the complexity of the matrices of patient samples could also be observed in the GC–MS data (Fig. 6). The total-ion chromatograms (TIC, acquisition of m/z ions between 33 and 400 amu) for the urine of which the CZE data are presented in Fig. 4 only shows two major peaks (panel A of Fig. 6). These peaks could be assigned to methadone (retention time 15.31 min) and M_1 (retention time 14.66 min) by comparing mass spectra obtained after extraction (Fig. 7) with those of a computer stored library of spectra containing data obtained after injection of model compounds. The library searches for methadone and M_1 resulted in recovery matches of 97 and 99%, respectively. The mass spectra shown in Fig. 7 also compare well with those reported in the literature [8]. The other example, which corresponds to that of Fig. 5, is characterized by a more complex TIC (panel B

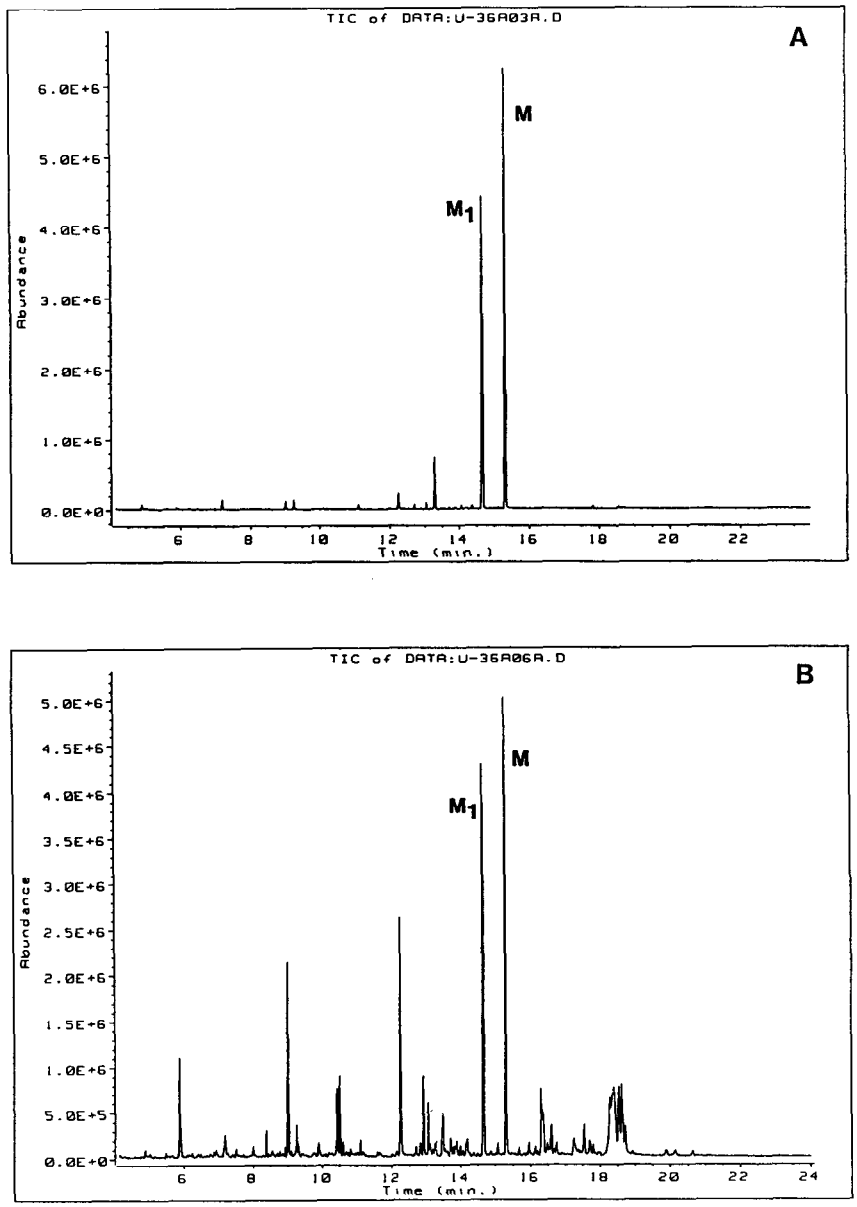

Fig. 6. GC–MS total-ion chromatograms of (A) patient urine No. 3, and (B) patient urine No. 6. Key as for Fig. 1.

of Fig. 6) compared to that of urine 3 (Fig. 6A), this paralleling the observations made by CZE. For this urine, methadone and M_1 could be clearly assigned as well. The recovery matches for methadone and M_1 were 95 and 99%, respectively.

For all 8 urines, the presence of methadone and M_1 in the extracts could be confirmed using both techniques, GC–MS and CZE with on-column multiwavelength absorbance detection. Thus, the presented data demonstrate the possibility to use CZE as an attractive alternative to customary GC–MS. The CZE data revealed that M_1 typically produced a higher peak than

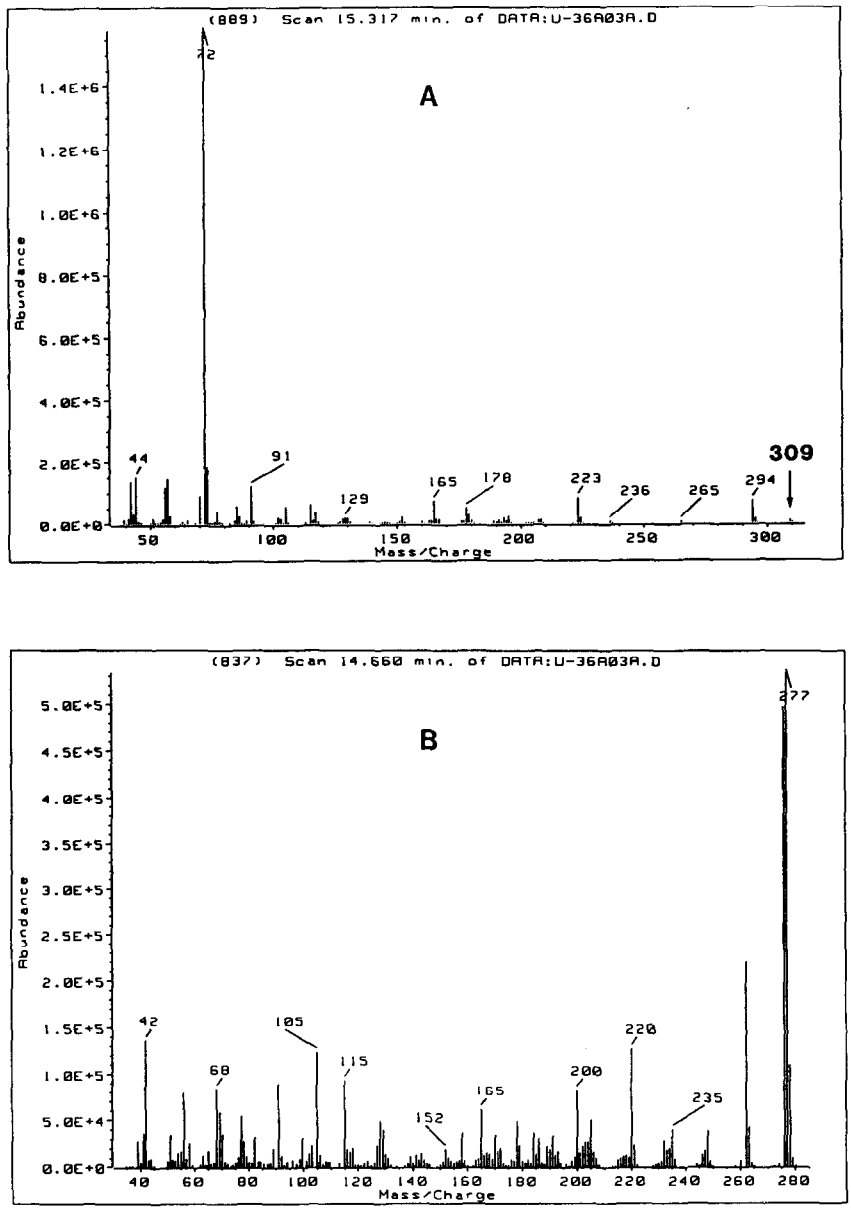

Fig. 7. Fragmentation data (mass spectra) of (A) methadone, and (B) M_1 as extracted from the data of Fig. 6A. The molecular ion of methadone with m/z of 309 is barely detected (panel A), whereas that for M_1 with m/z of 277 has a strong abundance (panel B).

methadone, this being in agreement with previous observations made on urines of patients under regular methadone intake (M_1/methadone ratio > 1 [5]). Using GC–MS, however, the opposite was found (Fig. 6), this being attributed to differences in extraction efficiency and/or detection discrimination. Using GC–MS, no quantitation was performed.

3.5. Drugs interfering with the CZE assay for methadone and M_1

Interferences originating from other drugs which coextract with methadone and M_1 represent a potential source of error. Although morphine, codeine, benzoylecgonine, and different benzodiazepine metabolites (3-hydroxybromazepam, 7-aminoclonazepam, desmethyldiazepam, 7-aminoflunitrazepam and α-hydroxymidazolam) are known to coextract with methadone and M_1, these compounds were found to appear around or after the electroosmotic system peak, thereby not interfering with methadone and M_1. Diphenhydramine, amphetamine and metamphetamine, however, also coextract and can be analyzed cationically by CZE at alkaline pH [15]. With the pH 9.3 buffer employed above, diphenhydramine was monitored distinctly after methadone (data not shown). Amphetamine was detected between methadone and M_1, whereas metamphetamine could not be separated from M_1 (Fig. 8A). This is not a problem in confirmation tests for methadone alone, but interferes with the screening for M_1 or metamphetamine. Decreasing the buffer pH to ca. 8.9 via addition of H_3PO_4, on the other hand, revealed baseline-resolved peaks for the four compounds (Fig. 8B) and thus permits the determination of methadone and M_1 in the presence of the two amphetamines; this is important for methadone compliance. Interference by the other compounds listed above was not observed at this pH. It is important to mention that pH selection was found to be critical and should be carefully investigated when employing this assay.

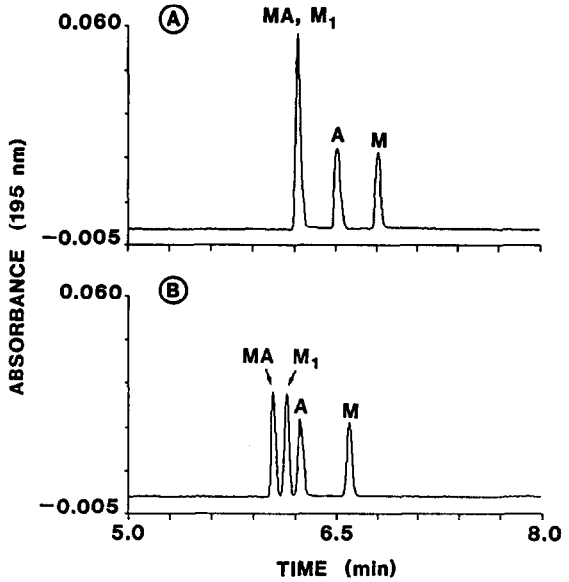

Fig. 8. CZE data of a standard mixture containing amphetamine (peak A), metamphetamine (peak MA), methadone (M) and M_1 using a tetraborate buffer of (A) pH 9.3 and (B) pH of 8.9. Other conditions as for Fig. 3.

3.6. Reproducibility and automated CZE assay

With the BioFocus 3000 device, the 8 urine samples were screened for methadone and M_1 using the two buffers with different pHs. Data obtained were essentially identical to those reported above for the homemade instrument, the exception being that somewhat shorter run times were obtained (Fig. 9). Reproducibility evaluations could easily be performed with this automated instrument. The data presented in Fig. 9 were obtained via direct injection of two urines, urine 3 at pH 9.3 (panels A and B of Fig. 9) and urine 8 at pH 8.9 (panels C and D). From 10 consecutive injections made with each sample, the first (panels A and C, respectively) and tenth (panels B and D, respectively) electropherograms are presented, and the data are shown to compare well. Calculated R.S.D. values for detection times, peak areas and peak heights of methadone and M_1 (for urine 3: 0.9 and 0.9%, 6.0 and 6.4%, and 6.4 and 5.3%, respectively; $n = 10$) further reveal the good reproducibility obtained with this assay performed on the BioFocus 3000 instrument. The three main features of this instrument, good reproducibility, automated operation and multiwavelength detection, provide the prerequisites for automated large scale urine screening. With appropriate software and a computer library of normalized absorbance spectra, automated CZE has the potential of becoming a simple and therefore attractive alternative to a similar toxilogical

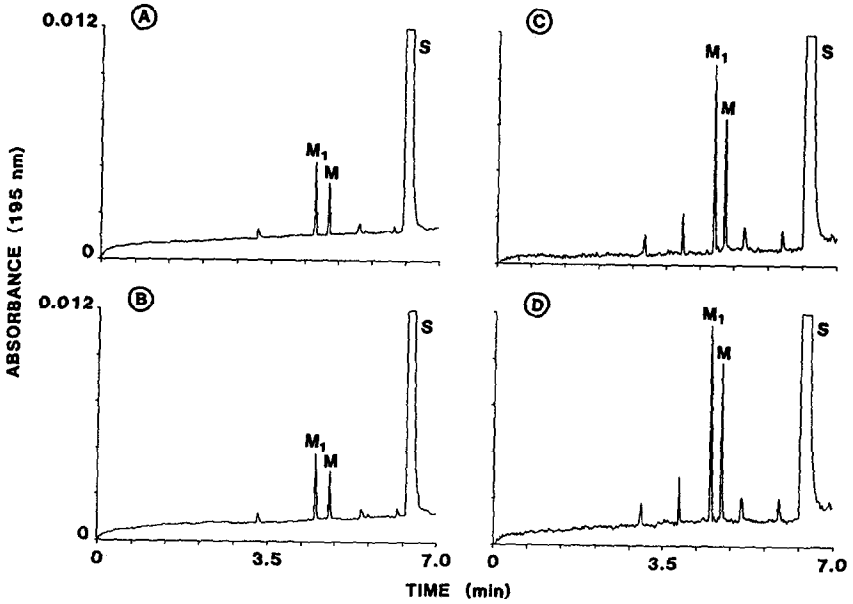

Fig. 9. CZE data obtained on the BioFocus 3000 after direct injection of (A, B) urine No. 3 using a buffer pH of 9.3, and (C, D) urine No. 8 employing a buffer pH of 8.9. The electropherograms depicted correspond to the first (panels A and C) and tenth (panels B and D) injection of 10 consecutive runs with each sample. The data presented in panels A and B were obtained via single-wavelength monitoring whereas those depicted in panels C and D were measured in the fast scanning mode. For the latter case, absorbance data at 195 nm are displayed only. Other experimental conditions are given under Experimental.

screening and confirmation approach previously developed on the basis of high-performance liquid chromatography [9].

4. Conclusions

CZE is shown to provide a simple and rapid approach for the determination of methadone and its primary metabolite in urine. This is in contrast to MECC in an SDS containing buffer, which does not permit an effective determination of these two compounds. CZE with on-column multiwavelength absorption detection is shown to allow analysis of these compounds in urine at the 2 μg/ml or higher level (direct injection of untreated urine), or at concentrations as low as 20 ng/ml (after solid-phase extraction using copolymeric sorbents). With CZE and solid-phase extraction, the presence of methadone and M_1 could be confirmed in all 8 investigated patient urines, urines which tested markedly positive for methadone using EMIT and in which the presence of methadone and M_1 was confirmed by GC–MS. Thus, there is good agreement between electrokinetic capillary data and data produced by GC–MS. For 6 of these urines, good agreement was found between CZE and direct urine injection. In two electropherograms, however, interferences of unknown origin (possibly originating from the complex urine matrix) made direct identification of methadone and M_1 impossible. Thus, for unambiguous characterization of the presence of methadone and/or M_1 by CZE, solid-phase extraction of the urines is preferred. With the exception of metamphetamine which coelutes with M_1, many other drugs (including amphetamine, diphenyhydramine, benzoylecgonine, opioids and benzodiazepines) which coextract with methadone, are shown not to interfere with the CZE assay performed at pH 9.3. Lowering the pH to 8.9

allows the separation of methadone, M_1 and the two amphetamines. Furthermore, good reproducibility of the CZE assay is documented.

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