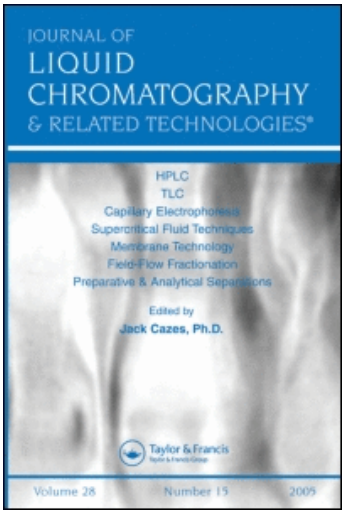

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The Determination of Methadone and Metabolites in Human Urine by HPLC with Ultraviolet, and Particle Beam Mass Spectrometric Detection

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THE DETERMINATION OF METHADONE AND METABOLITES IN HUMAN URINE BY HPLC WITH ULTRAVIOLET, AND PARTICLE BEAM MASS SPECTROMETRIC DETECTION

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ABSTRACT

Liquid chromatography coupled to Mass Spectrometry has been used to identify and quantitate methadone and two of its metabolites following a normal phase separation on a cyano-phase column. Solvent extraction was used to recover the target compounds from urine at the optimal pH of 10, (recovery: 88%, methadone; 75%, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). Routine analysis may be performed using UV detection at 290 nm. However, for greater selectivity and identification of eluates mass spectrometry is preferred. In the urine samples studied the methadone/EDDP ratio varied from 1:1 to 1:10. Two samples showed evidence for the presence of 1,5-dimethyl-3,3-diphenylpyrrolidone (DDP). Methadone may be

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detected selectively at m/z = 223 but the ion abundance is weak. Detection using the base peak m/z = 72 is preferred because of the increased sensitivity it confers although selectivity of detection is lost.

INTRODUCTION

In 1965 Dole and Nyswander (1) reported the importance and effectiveness of methadone as a treatment for opiate dependence. Since that time, methadone maintenance treatment has become one of the most widely used procedures for opiate dependency treatment. This application generated the need to understand the metabolism of methadone and to monitor the concentration of the drug and its metabolites in vivo. Various chromatographic techniques have been employed including thin layer chromatography (2), gas liquid chromatography (3), gas chromatography-mass spectrometry (4), radioimmunoassay (5) and enzyme immunoassay (6) techniques. More recently HPLC has been used (7) to study methadone metabolism in dogs but metabolite identity was presumed rather than established. An improved method based on HPLC for the determination of methadone and two major metabolites in rat plasma has also appeared (8). Separation is achieved on a C_{18} reversed phase column with an acetonitrile/water mobile phase. Solid phase extraction was used for preparation of the samples and gave excellent recoveries (85-100%). Difenoxin was used as internal standard. Studies of methadone metabolism in humans are less common. However, some results have appeared. For example the influence of liver disease on methadone metabolism has been assessed (9) and found to be negligible. Recent publications have tended to concentrate on the use of methadone to counteract opiate addition with particular emphasis being given to the measurement of the concentration of methadone itself in plasma (10,11). However this method utilises a silica column and UV detection at 215 nm.

Chromatographically silica columns have some disadvantages. Control of activity of the silica surface may be important to the separation and the carry-over of small amounts of water from the sample preparation stage may affect column activity. Pre-saturation of the silica surface with water mitigates this effect. Similarly, presaturation reduces the potential of silica to irreversibly adsorb material at the top of the column. Use of the low detection wavelength (215 nm) is relatively non-specific and may yield a relatively high signal-to-noise ratio. However, this method has been used successfully to study the steady-state pharmacokinetics of methadone in opioid addicts (12).

A major factor influencing methadone metabolism is reported to be the co-administration of other drugs. Methadone kinetics have been reported to be disrupted by carbamazepine (13), barbiturates (14) and amitriptyline (15). Hence methods which identify and quantitate both the parent compound and the major metabolites are needed to allow variation in the metabolism of methadone to be studied We now report some preliminary results on the use of HPLC coupled to particle beam mass spectrometry to identify metabolites of methadone extract from the urine of patients undergoing methadone therapy.

EXPERIMENTAL

Materials

All solvents were of HPLC grade and ammonium acetate was AnalaR grade (BDH Chemicals Ltd). Distilled water was checked for UV absorbence (200 nm - 300 nm) before use. Racemic d, l - methadone (Sigma Chemicals, Poole, Dorset) and EDDP (donated by Leeds Hospital) were used as received.

Equipment

A Philips Analytical PU4015 dual piston pump (Philips Analytical, Cambridge UK), a UV detector (PU4025), (Philips Analytical, Cambridge, UK) fitted with an 8 µl flow cell (10 mm path length) and an integrator (HP3394, Hewlett Packard) were used. The column was Nucleosil 5CN (250 mm x 4.6 mm id) (Phenomenex Cat No OOG-0324-EO) and injection (20 µl or 100 µl) was via a Rheodyne 7010 valve. The mobile phase was 30% 0.1M ammonium acetate adjusted to pH 3.6 with glacial acetic acid: 70% acetonitrile. Batches of mobile phase were degassed with helium (20 minutes) before use. A detector wavelength of 280 nm and a flow rate of 1.0 ml min^{-1} for UV detection and 0.6 ml min^{-1} for mass spectrometric detection was used. The mass spectrometer (TRIO-1) and particle beam interface (LINC type) were supplied by VG MASSLAB Ltd (Crewe Road, Wythenshawe, Manchester M23 9BE, UK).

Extraction Procedures

The pH of 10 ml urine was adjusted to 9.5 - 10.0 with ammonium hydroxide. A 1 ml aliquot was then mixed with 5 ml of 1-chlorobutane (Aldrich Chemical Co Ltd, The

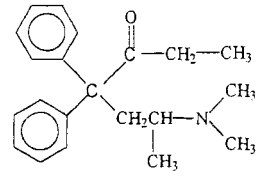
Old Brickyard, New Road, Gillingham, Dorset, SP H4JL) and the sample shaken mechanically for 30 minutes. Centrifugation (2000 rpm, 5 mins) yielded a 1-chlorobutane top layer which was removed to a clean glass vial. 20 µl of dimethylformamide was added to act as a "keeper" solvent to minimise loss of methadone during evaporation of 1-chlorobutane under a stream of dry nitrogen. The residue sample (volume approximately 20 µl) was stored at O ^{o}C until reconstituted in the mobile phase (500 µl) immediately prior to injection.

RESULTS AND DISCUSSION

Methadone (I) is extensively metabolised in the body by a variety of pathways. For subjects on a methadone maintenance programme the ratio of the primary metabolite (EDDP) to methadone is normally much higher than in an overdose case. The primary metabolic reaction in man is oxidative N-demethylation to form an intermediate (N-desmethyl -methadone) which spontaneously cyclises to form EDDP (II), which thus contains a pyrrolidine structure. A diphenyl methyl moiety is common to both structures and provides the major source of uv absorbence albeit a relatively weak one due to non-conjugation. Subsequent reactions include hydroxylation of a phenyl ring to yield hydroxy-EDDP (III) which now contains a phenolic function, and demethylation to provide EMDP (IV). Another pathway involves ketone oxidation and oxidative removal of the ethyl group on methadone followed by spontaneous cyclisation to form DDP (V) which contains a pyrrolidone structure.

Chromatography

Polar drugs and their metabolites are usually separated by reversed phase chromatography. The literature indicates that a cyano-bonded phase is most appropriate for methadone and was selected for this work. Selection of the mobile phase was restricted by the need for compatibility with the particle beam interface. Hence, no involatile buffer salts could be used. Likewise, use of ion-pairing agents such as alkane sulphonic acids is excluded because of incompatibility with the use of mass spectrometry as the detection mechanism. The choice of a cyano-bonded phase however, renders their use unnecessary as the cyano group provides the polar functionality necessary for retention. Ammonium acetate was chosen as the buffer salt as it is volatile and hence amenable to use with the particle beam interface and mass spectrometry. It also contributes marginally to improved chromatographic efficiency by moderating peak tailing.



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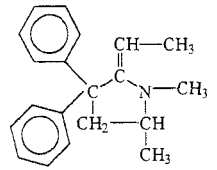

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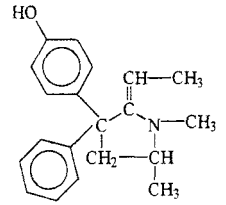

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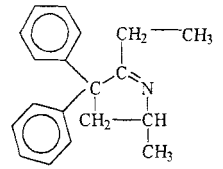

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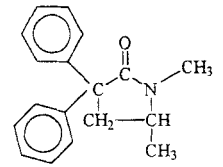

(V)

The most often selected wavelength for detection of methadone is 215 nm. However, given the much greater concentration of methadone present in urine we have investigated the use of higher wavelengths which have provided the added benefit of greater selectivity for our particular compounds of interest. Molar extinction coefficients are generally lower at higher wavelengths but signal-to-noise ratios are better and this fact may be exploited to regain sensitivity of detection. A UV spectrum of methadone in the mobile phase indicated two maxima at 259.8 nm and 293.6 nm of similar intensity [absorbence (259.8 nm) = 0.187, absorbence (213.6 nm) = 0.183, for a 1 mg in 10 ml solution]. The higher wavelength was chosen because of the increase in absorbence at 260 nm caused by the glacial acetic acid. Using the selected chromatographic conditions (1.0 ml min^{-1}, 70% acetonitrile: 30% ammonium acetate, pH = 3.6 with glacial acetic acid, 20 µl injection, 293.6 nm) the retention time for methadone was 5.35 min (k' = 4.94) and for EDDP was 5.70 min (k' = 5.33) for standards injected in the mobile phase. For spiked urine extracts a large amount of co-extracted material elutes at approximately 2.9-3.5 minutes and causes a slight shift of retention to 5.45 minutes (k'=5.05) and 5.90 (k'=5.55) respectively. The nature and complexity of the co-extracted material varies from sample to sample and this is reflected in small changes in retention time for the target analytes. The example shown in Figure 1 is a spiked urine sample which is particularly rich in co-extracted material yet still gives good resolution for methadone (t_R = 5.45, k' = 5.05) and EDDP (t_R = 5.89, k'=5.5).

For mass spectroscopic studies the flow rate was reduced to 0.6 ml min^{-1} to accommodate the capabilities of the particle beam interface. Both the EI and CI modes were evaluated. The mass range used was 65-500 amu. Methadone and EDP standards were used to generate spectra. Urine extracts from known methadone users were screened to attempt to identify other possible metabolites present. Using the EI mode methadone, EDDP and DDP (V) were identified but when CI(methane) was employed hydroxylated EMDP(III) was observed and DDP was not found. Figure 2 shows a summed ion intensity (m/z = 65:500) chromatogram obtained in the EI mode for a urine extract. Comparison of mass spectra obtained indicates that the peaks at t_R = 8.79, t_R 7.04 and t_R = 9.31 are methadone, DDP and EDDP respectively. Examination of the peaks at t_R = 6.16 and ca 8.26 indicate that they do not contain ions representative of other metabolites. Presumably these are co-extracted substances which are not uv responsive at 290 nm (Figure 1).

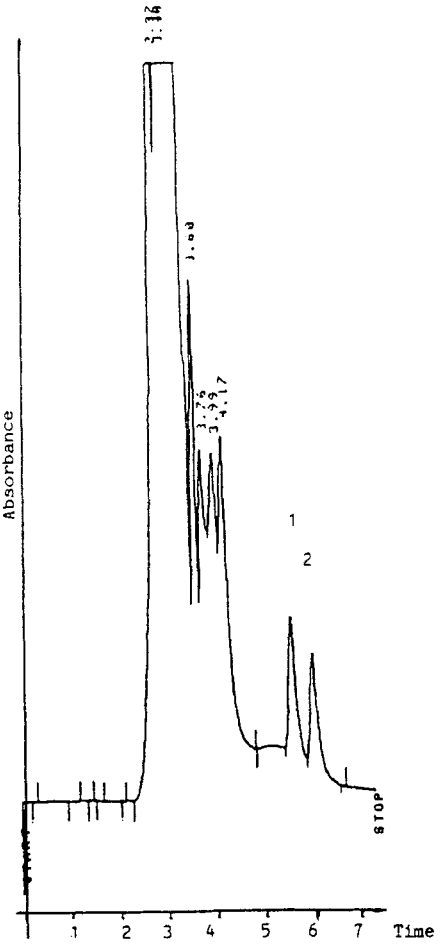

Figure 1 Urine sample spiked with methadone (40 mg l^{-1}, 0.129 mmol) and EDDP (40 ng l^{-1}, 0.144 mmol) chromatographed following extraction with n-butyl chloride, 1 = methadone, 2 = EDDP

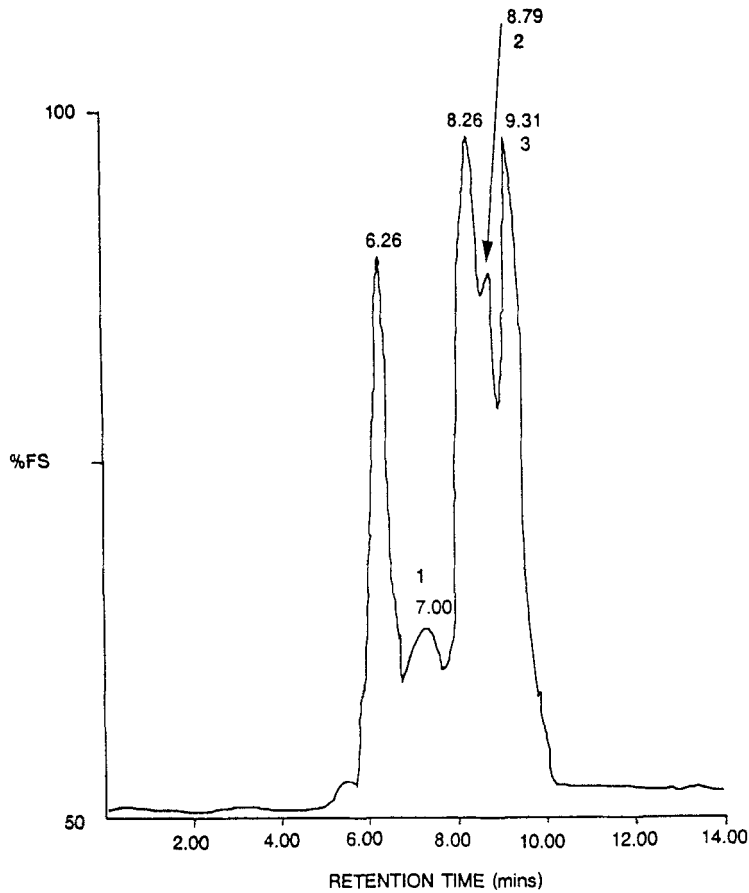

Figure 2 Summed ion intensity chromatogram obtained in EI mode for a urine extract 1 = methadone, 2 = DDP, 3 = EDDP.

Table 1 shows the groups of ions for the various compounds under study. In the EI mode, methadone gives a strong base peak at m/z = 72 corresponding to the $C_4H_{10}N$ fragment from the side chain. Methadone concentrations are frequently found to be too low to use the weak ion at m/z = 223 for monitoring as the EI abundance is only 1.65%. In order to enhance sensitivity the less selective but much more intense base peak (100% relative intensity) at m/z = 72 may be used. No parent ion for methadone (EI mode) at m/z = 310 was observed. Other fragments with masses of

Table 1 Suitable ions for selected ion monitoring for methadone and its major metabolites.

ANALYTE	MW	$[M+1]^+$ (CI)	MONITORING ION	OTHER IONS
METHADONE	310	311	72	223,265,165,179
EDDP	277	278	277	200,234,262,276,106
EMDP	263	264	263	208
HYDROXYLATED EDDP	293	294	-	-
DDP	265	266	265	194,179,130,115

165 and 179 appear to contain the diphenylmethyl moiety. EDDP shows a molecular ion at m/z = 277. The peak at m/z = 208 is reportedly weak (0.8%) and we confirm this observation. Loss of the sidechain is thus not a favoured process. The fragment of mass 106 appears common to both EDDP and DDP. No evidence for the presence of significant concentrations of other metabolites was found in the samples studied.

Calibration

Calibration curves were obtained for methadone and EDDP for use in recovery studies. External calibration was used and uv detection was employed. Calibration curves over the range 0 to 100 mg l^{-1} for both methadone (0-0.323 mmol) and EDDP (0-0.361 mmol) were obtained and found to be linear for both methadone (slope = 1.480 = intercept (y) 3.94 : correlation coefficient = 0.9998 and EDDP, (slope = 1.934: intercept (y) = 3.57, correlation coefficient = 0.9978). The calculated limit of detection with detection in the ultra-violet was ca 20 ng on column, as reported previously by Wolff et al. (11)

Recovery of Methadone and EDDP from Urine

Liquid-liquid extraction methods are most commonly used for the recovery of methadone from urine. Both methadone and its metabolites may be recovered from alkaline urine. A high pH suppresses the protonation of methadone and related compounds rendering them more readily partitioned into an organic solvent. Published extraction methods reveal the use of dichloromethane, hexane, ethylacetate and 1-chlorobutane as partitioning solvents. Attempts to achieve efficient recovery with hexane, dichloromethane and ethylacetate proved unsuccessful. Previous work with 1-chlorobutane gave mixed values for recovery with Wolff et al (11) reporting 98% recovery of methadone (range 90-110%) whereas Buice (16) claims only 63.5%. Sullivan and Blake (17) demonstrated that 1-chlorobutane was an effective extracting solvent. To optimise the extraction procedure, two blank urine samples were spiked with methadone to give concentrations of 1.37 mg l^{-1} (4.4 μmole) and 1.48 mg l^{-1} (4.8 μmole) respectively for recovery at low concentrations and 22, 40, 60 and 80 mg l^{-1} for recovery at high concentrations. Extractions were performed at five different pH values (2.0, 5.0, 8.0, 10.0, 12.0) by adjustment with either glacial acetic acid or ammonium hydroxide solution. 1-Chlorobutane was used as solvent. The extraction time (by shaking) was 30 minutes, followed by centrifugation (2000 rpm, 5 minutes)

and removal of the upper layer . Approximately 20 µl of dimethylformamide (DMF) was added as 'keeper' solvent to minimise loss of analytes during evaporation of the 1-chlorobutane under a gentle stream of dry nitrogen. The oily residue consisting of extracted material with ca 20 µl of DMF was stored at 0 °C until reconstituted with mobile phase immediately prior to analysis. The average recovery for methadone was 88% at pH, 10 with lower values for other pH values.

A similar process was performed for recovery of EDDP from urine spiked at 1.5 mg l^{-1} (5.4 µmole) and at 40 µg l^{-1} (144 µmole) and 60 mg l-1 (216 µmole). Again the pH was varied over the same range as for methadone with the best recovery being obtained at pH = 10 (75%). Other values obtained were 32.5% (pH=12), 42.5% (pH=8) and no detectable amount was recovered at pH2 and pH5.

It should be noted that whilst the extraction of methadone and EDDP is from alkaline urine, the chromatographic separation is carried out at pH 3.6. Furthermore, no methadone or EDDP was recovered into the organic phase at pH 2 or 5. Both methadone and EDDP can be classed as tertiary amines although the nitrogen atom in EDDP is contained in a 5-membered ring. Both compounds are thus likely to display basic characteristics and exist as free bases at the pH of extraction. At acidic pH however the lone pair on the nitrogen will protonate rendering both these structures cationic and thus highly polar. Hence there is a need to use an ion-pairing agent to obtain retention on a C_{18} bonded phase column. When a cyano-bonded phase is used the -C≡N group provides sufficient electron density to retain the protonated species. Hence retention is affected by both solvent composition and pH. In the separation system used it is the acetate ion which acts as the counter ion to the protonated compounds and thus duplicates the role of the ion pairing agent (normally an alkyl sulphonic acid group,) albeit with a smaller effect. This small effect does, however, contribute to an improvement in peak shape. Hence the cyano column
operates in a similar retention mode to a silica column (11) but suffers less from irreversible adsorption.

Application to Urine Samples

A total of 16 samples was obtained from a drug therapy clinic. Each sample was extracted using the procedure based on 1-chlorobutane and analysed using both UV and MS detection. Of these 16 samples, 8 revealed no detectable amounts of either methadone or any identifiable metabolite. Of the 8 positive samples all contained

measurable concentrations of both methadone and EDDP. The ratio of the concentrations of methadone to EDDP ranged from <1:1 (3 samples) through an approximate 1:1 ratio (2 samples) to 1:10 (3 samples). These results tend to confirm that methadone metabolism is individually defined. In addition, two samples studied showed evidence (m/z =265) for the presence of DDP. The mass chromatogram (Figure 3) shows the response for DDP at t_R=7.00. The peak at t_R = 6.23 in the ion chromatogram for m/z =72 is not thought to be related to methadone and serves as a reminder of the non-specificity of this ion.

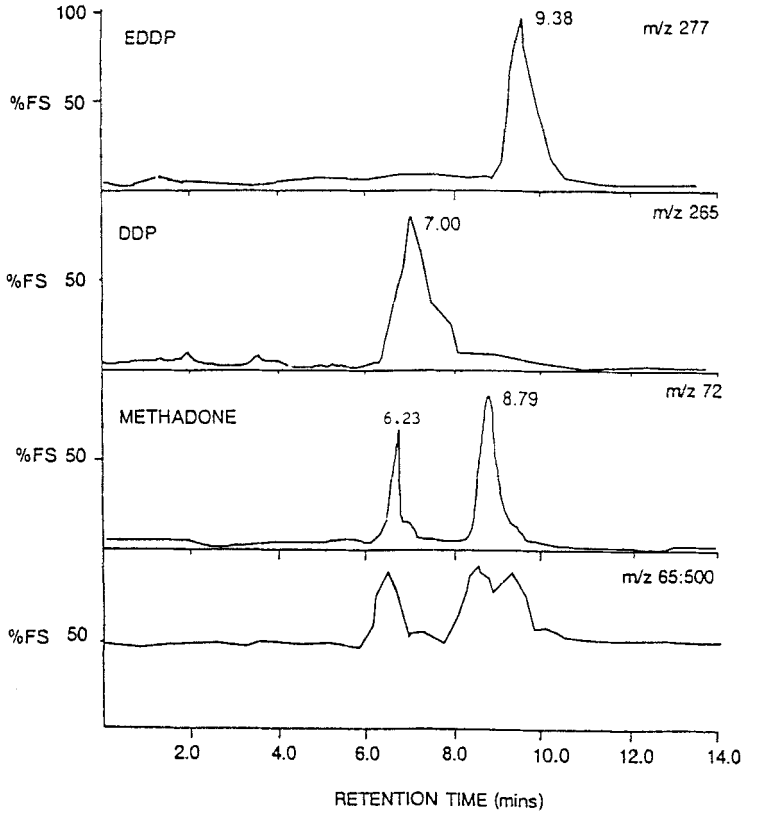

Figure 3 Mass chromatograms obtained in EI mode for a human urine extract indicating the presence of DDP (t_R = 7.00m, m/z = 265) in addition to EDDP (t_R = 9.38m m/z = 377) and methadone (t_R = 8.79m, m/z = 72).

CONCLUSIONS

Methadone and EDDP may be recovered from alkaline urine using 1-chlorobutane as solvent with recoveries of 88% and 75% respectively.

Separation on a cyano-bonded column is achieved with uv detection at 290 nm or by particle beam mass spectrometry. Of 16 samples analysed, 8 were found to contain methadone and EDDP and two of these revealed evidence of an additional metabolite DDP but no standard was available for quantitation. Although analysis may be carried out by using UV detection, mass spectrometry provides useful information about the identity of co-extracted material and provides evidence of identity which can indicate the presence of additional metabolites.

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