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# DETERMINATION OF THE HEROIN METABOLITE 6-ACETYLMORPHINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AUTOMATED PRE-COLUMN DERIVATIZATION AND FLUORESCENCE DETECTION

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## SUMMARY

An improved method for the determination of 6-acetylmorphine in the urine of drug addicts receiving morphine was developed. A newly introduced reversed-phase high-performance liquid chromatographic system proved to be more sensitive than a normal-phase system used previously. By replacing the earlier manual derivatization procedure with an automated on-line pre-column method, both the reproducibility and efficiency were considerably improved. Coefficients of variation for repeated analyses typically ranged from 6 to 10% in the 1–100 μg/l concentration range. The detection limit was 1 μg/l and the correction for recovery by calibration with blank urine samples spiked with 6-acetylmorphine was satisfactory. The analytical improvements achieved, however, did not increase the chance of detecting heroin use by drug addicts.

## INTRODUCTION

For more than 2 years the Municipal Health Department of Amsterdam has been supplying a limited number of extremely problematic drug addicts with daily rations of injectable morphine. This treatment programme is closely monitored with, among other methods regular urine analyses to detect possible continuation of heroin use by the drug addicts.

Heroin use is most conveniently detected by determining its principal metabolites morphine and morphine-3-glucuronide in urine[1], but this technique is obviously useless in this instance. The only specific marker for heroin use is 6-acetylmorphine, which, unfortunately, is only a minor urinary metabolite as it is further metabolized to morphine to a large extent[2]. To have a reasonable chance of detecting heroin abuse under these conditions, the method used should be extremely sensitive. Previously, high-performance liquid chromatographic (HPLC) method with fluorescence detection was developed[3], based on mild oxidation of 6-acetylmorphine in the

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presence of excess of morphine to yield a highly fluorescent condensation product. The reaction products were separated by normal-phase HPLC, which allowed the detection of approximately 5 μg/l of 6-acetylmorphine in urine. In this paper, an improved method consisting of reversed-phase HPLC and automated pre-column derivatization is presented. The main incentives to improve the method were, in order of importance, (i) the notion that lowering the detection limit could possibly improve the chance of detecting heroin use; (ii) the limited reproducibility of the method, which was mostly due to the instability of the 6-acetylmorphine/morphine condensation product causing the samples to deteriorate slowly while standing in the autosampler; and (iii) the detrimental effect of the solvent used on the piston seal of the HPLC pump.

The new method was compared with radioimmunochemical methods for both total and free morphine to establish the relative efficiencies of these methods in detecting heroin use.

## EXPERIMENTAL

### *Materials and equipment*

All solvents and reagents were of analytical-reagent grade. Water was distilled twice in Pyrex glass. 6-Acetylmorphine was prepared from morphine as described previously[3]. HPLC columns (150 × 4.6 mm I.D.) were packed with Hypersil ODS 5 μm (Shandon, Runcorn, U.K.) by means of a Shandon slurry packer and according to the manufacturer's instructions. The chromatograph consisted of a Kipp Analytica (Emmen, The Netherlands) Model 9208 pump, an LKB (Bromma, Sweden) 2153 Autoinjector, a laboratory-built column thermostat and a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 650-10LC fluorescence spectrometer.

### *Methods*

Urine samples were extracted as described previously[3]. Briefly, the method consisted of extraction with 15% (v/v) 2-propanol in dichloromethane on an Extrelut

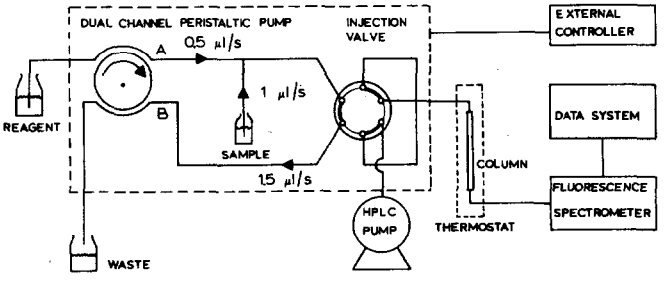


Fig. 1. Automatic pre-column derivatization device of the LKB 2153 Autoinjector as used in 6-acetylmorphine derivatization. The derivatization–injection procedure is initiated by switching the injection valve into the "load" position and starting the peristaltic pump. Channel B of the pump draws the reagent–sample mixture through the sample loop at a rate of 1.5 μl/s. Channel A delivers reagent at a rate of only 0.5 μl/s so that sample is drawn at a rate of 1 μl/s. After 150 s the pump is stopped and the reaction mixture is left in the sample loop for 120 s. Then the valve is switched into the "inject" position and the contents of the sample loop are transferred to the column.

column, followed by back-extraction of the organic solvent with dilute sulphuric acid. The latter was rendered alkaline and extracted with 2-propanol–dichloromethane as described above.

After evaporation of the organic solvent, the residue was dissolved in 200 μl of a 125 mg/l solution of morphine hydrochloride in 0.015 mol/l hydrochloric acid, then 10 μl of 0.3 mol/l Tris buffer (pH 8.5) were added to the samples, which were subsequently placed in the autosampler. The latter was equipped with a pre-column derivatization device as depicted in Fig. 1. The reagent delivered by pump A was a 0.015 mol/l solution of potassium hexacyanoferrate(III) in water. The mixing ratio of sample and reagent was 2:1. The injection cycle was initiated by flushing the 50-μl sample loop with sample–reagent mixture for 150 s. The reaction was then allowed to proceed for 120 s, after which the contents of the sample loop were injected immediately. Separation was accomplished using the solvent system 0.1% (v/v) triethylamine in acetonitrile–water (16:84, v/v) at a flow-rate of 1.5 ml/min. The column temperature was maintained at 30°C. Under these conditions, the back-pressure was 150 bar and the retention time of the 6-acetylmorphine/morphine dimer was approximately 10 min. The column eluate was fed into the fluorescence spectrometer, the excitation and emission monochromators of which had been adjusted to 320 and 436 nm, respectively, with a 10 nm bandwidth. Between the chromatographic runs three consecutive injections were made from a vial containing 0.1% of triethylamine in acetonitrile–water (40:60, v/v) to prevent carry-over between samples. Quantitation was based on peak-height measurement and corrections for recovery were made using the external standard method.

In one experiment both free and total morphine were determined in urine samples by radioimmunochemical methods. For total morphine the Abuscreen kit from Hoffman-LaRoche (Nutley, NJ, U.S.A.) and for free morphine the Coat-a-count kit from Diagnostic Products (Los Angeles, CA, U.S.A.) were used.

## RESULTS AND DISCUSSION

The first aspect of the revised method to be investigated was the possible occurrence of compounds in urine samples that would coelute with the 6-acetylmorphine/morphine condensation product. This had been the sensitivity-limiting factor in the normal-phase HPLC system used previously. Therefore, six blank urine samples were obtained from laboratory staff members and were subjected to the new method. In the chromatograms obtained no peaks at or near the retention time of the 6-acetylmorphine/morphine condensation product could be observed and it was concluded that the new method might offer the desired higher sensitivity.

It was felt that the new automatic pre-column derivatization device might give rise to considerable carry-over between samples. As the 6-acetylmorphine concentration of the urine samples to be analysed varied over four orders of magnitude, any carry-over would be unacceptable. By analysing highly concentrated and blank urine samples in turn it was found indeed that considerable carry-over occurred. The LKB Autoinjector used did not offer convenient facilities for flushing between samples but by connecting it to an external programmable timer, any number of flushing cycles could be applied. I was found that the flushing conditions mentioned above reduced carry-over to undetectable levels.

TABLE I

REPRODUCIBILITY OF 6-ACETYLMORPHINE DETERMINATIONS IN ENRICHED URINE SAMPLES

| *6-Acetylmorphine concentration (μg/l)* | | | | | *Mean squares* | | *F* | *p* |
|---|---|---|---|---|---|---|---|---|
| *Calculated* | *Found* | | | *Mean* | *Between results* | *Among results* | | |
| | *Week 1* | *Week 2* | *Week 3* | | | | | |
| 5 | 4.35 | 4.29 | 3.93 | 3.91 | 0.31 | 0.05 | 6.0 | <0.05 |
| | 3.87 | 3.93 | 3.75 | | | | | |
| | 4.03 | 3.93 | 3.40 | | | | | |
| | 4.03 | 4.11 | 3.40 | | | | | |
| | 3.55 | 4.29 | 3.57 | | | | | |
| | 3.87 | 4.11 | 3.93 | | | | | |
| 25 | 18.40 | 19.23 | 18.58 | 18.27 | 1.58 | 0.35 | 4.5 | <0.05 |
| | 19.45 | 18.00 | 18.23 | | | | | |
| | 18.40 | 18.53 | 16.78 | | | | | |
| | 18.40 | 17.83 | 17.85 | | | | | |
| | 18.40 | 18.88 | 16.78 | | | | | |
| | 18.40 | 18.88 | 17.85 | | | | | |

To validate the method further, its linearity, reproducibility, recovery and sensitivity were investigated. Solutions of 20, 40, 100, 200, 500, 1000 and 2000 μg/l 6-acetylmorphine in methanol were prepared. As the concentration factor of the extraction procedure was 20, these concentrations corresponded to urine concentrations ranging from 1 to 100 μg/l. From each of these, 33.3 μl (sample loop volume 50 μl, dilution factor 1.5) were injected on the column twice. Linear regression of the 6-acetylmorphine peak heights obtained to the corresponding equivalent urine concentrations gave the regression equation $Y = 4.30X - 6.35$, where $Y$ = peak height (mm) and $X$ = concentration (μg/l). The correlation coefficient was 0.9941, the residual standard deviation was 16.99 and the standard deviations of the regression coefficient and intercept were 0.13 and 5.87, respectively.

To investigate both reproducibility and recovery, a blank urine pool was divided into two portions, which were enriched with 5 and 25 μg of 6-acetylmorphine per litre of urine, respectively. Both pools were analysed six times on each of three occasions with intervals of approximately 1 week. The results from these experiments were used to calculate the reproducibility and, by comparison with concurrently analysed standard solutions, the analytical recovery for both concentration levels. The results are presented in Table I. There was a significant variation between analytical series, as could be shown by an analysis of variance ($p < 0.05$ for both levels). The recoveries calculated from these data averaged 78 and 73% for the 5 and 25 μg/l samples, respectively. As the recoveries were obviously subject to the same between-run variation as mentioned above, it was found useful to include recovery checks in each run. Because the reproducibility data in Table I did not apply to such conditions, another experiment was devised. In this experiment a urine pool was prepared by combining urine samples from heroin addicts and dividing the mixture into eight portions, which were diluted with varying amounts of a blank urine pool. The 6-

TABLE II

REPRODUCIBILITY OF 6-ACETYLMORPHINE DETERMINATIONS USING ENRICHED BLANK URINE SAMPLES AS STANDARDS

| *Urine pool No.* | *6-Acetylmorphine concentration (μg/l)* | | |
|---|---|---|---|
| | *Calculated** | *Average found*** | *Coefficient of variation (%)* |
| 1 | 95.3 | 94.1 | 7.0 |
| 2 | 76.2 | 63.3 | 6.0 |
| 3 | 63.5 | 57.5 | 8.3 |
| 4 | 42.3 | 40.9 | 7.8 |
| 5 | 21.2 | 19.4 | 9.3 |
| 6 | 10.6 | 10.1 | 13.9 |
| 7 | 5.3 | 5.2 | 9.6 |
| 8 | 2.1 | 2.0 | 10.0 |

* Calculated from the 6-acetylmorphine concentration of the original urine pool that was used to prepare pools 1–8.

** Average of six determinations equally distributed over three analytical runs.

acetylmorphine concentrations in the urine pools so obtained ranged from 2 to 94 μg/l. Urine samples taken from these pools were analysed in duplicate on three different occasions at 1 week intervals. In this instance the peak heights were compared with those obtained from two blank urine samples enriched with 25 μg/l of 6-acetylmorphine. Table II shows the mean concentrations found and those calculated from the concentration as determined in the original urine pool. With one exception, which was probably due to a dilution error, the concentrations found agreed well with those calculated. The coefficients of variation generally were between 6 and 10% with a slight but statistically significant (linear regression: hypothesis $\rho = 0$; $p < 0.05$) increase with decreasing concentration. However, even at the lower end of its dynamic range the method seemed to offer good reproducibility.

The absolute detection limit was 0.25 ng injected on the column (signal-to-noise ratio = 2). When two blank urine samples were enriched with 2 and 1 μg/l of 6-acetylmorphine, easily recognizable peaks were obtained with signal-to-noise ratios of 4 and 2, respectively.

The results mentioned above showed that the analytical aims set had been achieved, but it remained to be demonstrated that the improvements did indeed increase the chance of detecting heroin use. For obvious reasons, it was impossible to test this under experimentally satisfactory conditions. Therefore, it was decided to repeat an experiment carried out previously[3] with the use of a normal-phase HPLC system. In this experiment urine samples had been collected at random from 50 heroin addicts who did not receive morphine and these samples had been shown to contain at least 0.4 mg/l of total morphine by a standard enzyme mediated immunochemical (EMIT) method. In 72% of the samples 6-acetylmorphine had been detectable with reasonable confidence.

Again, 50 urine samples were collected from heroin addicts but in this instance both the free and total morphine concentrations were determined by radioimmu-

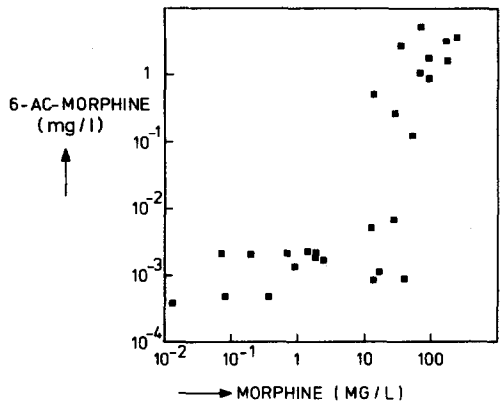


Fig. 2. 6-Acetylmorphine concentrations *versus* total morphine concentration in 28 urine samples collected from heroin addicts.

noassay. The 6-acetylmorphine concentrations were determined subsequently, and the results compared with the total morphine concentrations. In Fig. 2 the 6-acetylmorphine concentrations are plotted against the total morphine concentrations for all 28 samples in which both compounds were detectable. These samples amounted to 56% of those analysed, but this figure cannot be compared directly with the 72% found using the normal-phase HPLC method because, as was shown by radioimmunoassay, only 36 samples contained at least 0.4 mg/l of total morphine. When only the latter samples were taken into consideration, 64% of them were found to contain detectable levels of 6-acetylmorphine, a number fairly close to the 72% found previously. From Fig. 2 it is clear that no simple relationship exists between the concentrations of the two compounds and that the 6-acetylmorphine concentration tends to decrease much faster than that of total morphine. A similar picture emerged when the 6-acetylmorphine concentration was related to that of free morphine. In contrast, the concentration of the latter correlated excellently with that of total morphine [linear regression: $Y = 0.063X + 0.121$, where $Y$ and $X$ are the concentrations of free and total morphine (μg/l), respectively; correlation coefficient, 0.964; residual standard deviation, 1.730]. Apparently, 6-acetylmorphine is eliminated from the body more rapidly than morphine, a conclusion that is supported by pharmacokinetic studies carried out in dogs[4]. From this the conclusion can be drawn that increasing the sensitivity beyond what has been achieved in the method presented above does not contribute to a better chance of detecting heroin use.

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REFERENCES

1 U. Boerner, S. Abbott and R. L. Roe, *Drug Metab. Rev.*, 4 (1975) 39–73.
2 H. W. Elliot, K. D. Parker, J. A. Wright and N. Nomof, *Clin. Pharmacol. Ther.*, 12 (1971) 806–814.
3 H. J. G. M. Derks, K. van Twillert and G. Zomer, *Anal. Chim. Acta*, 170 (1985) 13–20.
4 E. R. Garret and T. Gurkan, *J. Pharm. Sci.*, 69 (1980) 1116–1134.