ELSEVIER

Journal of Chromatography B, 663 (1995) 225–233

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Analysis of common opiates and heroin metabolites in urine by high-performance liquid chromatography

A.S. Low, R.B. Taylor*

School of Pharmacy, The Robert Gordon University, Schoolhill, Aberdeen AB9 1FR, UK

First received 1 June 1994; revised manuscript received 20 September 1994

Abstract

A separation of heroin, 6-monoacetylmorphine, codeine, pholcodine, dihydrocodeine and morphine using a 200×2 mm I.D., 3 μm silica column with dichloromethane–pentane–diethylamine–methanol mobile phase is described. Data on the determination of these compounds in a urine matrix based on this separation using a solid-phase pretreatment with Bond Elut Certify cartridges and nalorphine as an internal standard are shown. The compounds listed can be quantified at levels below that generally accepted as the cut-off level for the screening for opiates by enzyme immunoassay (EMIT) with detection limits for the different opiates ranging from 4 to 20 ng ml^{-1}. Comparative data are shown of subject urine samples assayed for opiates by both the enzyme immunoassay and the proposed method. The utility of the method for the elimination of so-called false positives detected by EMIT due to the presence of medically prescribed and non-prescription opiates in urine is discussed.

1. Introduction

Several publications have appeared in the literature dealing with the development of liquid chromatographic methods of detection and quantification of certain of the opiate drugs in biological matrices such as plasma and urine [1–14]. The purpose of some of these publications [3,7,11] is stated as providing an alternative confirmation assay method for opiates to the conventionally accepted methods based on GC–MS. The majority of the publications located are concerned with the measurement of concentrations of morphine and its conjugated metabolites, [2,3,13], sometimes in conjunction with codeine [4,7,11]. Two publications deal with the determination of pholcodine and its metabolites in urine and whole blood respectively [8,10]. The application of such methods is limited, since there is little if any indication of the utility to determine other members of this class of drugs. In contrast, there are few publications which describe methodology for the simultaneous determination of a range of opiates. One such report describes different solvent systems for the identification of all the major classes of abused drugs [14]. However this is not quantitative in the case of the opiates and only separations of codeine and morphine are shown. Another, while describing separations of several opiates, is concerned with adulterants of heroin in solid samples [5].

Following a positive immunoassay test for opiates in a drug screening programme it is usual

* Corresponding author.

0378-4347/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0378-4347(94)00459-5

to determine the particular opiates present in the sample. Heroin and codeine are both metabolised to morphine, although at different rates, and the value of the codeine/morphine ratio is very generally used, together with the concentration of morphine determined, to attribute the presence of morphine to the consumption of heroin (or morphine itself) or to codeine. While codeine is the major legal opiate detected during drug screens, other generally used drugs such as pholcodine and dihydrocodeine undergo similar metabolism. The benchmark analytical method for the specific detection of opiates in these situations is GC–MS. This technique is not, however, generally accessible and is still expensive. Alternative, more readily available, analytical procedures allowing determination of the above parameters of morphine concentration and legal opiate/morphine ratio would, therefore be of value. Such methods should be specific for the drugs to be determined, without interference from heroin and the 6-monoacetyl metabolite. To our knowledge no such method utilising the advantages of liquid chromatography, i.e. ease of operation and general availability, has yet been published. It is the purpose of the present paper to describe an HPLC method for determination of opiates which is relevant in this case and to demonstrate its quantitative application to the determination of specific opiates in urine samples.

2. Experimental

2.1. Materials

Heroin (diacetylmorphine) was obtained from DM Wood (Aberdeen, UK), 6-monoacetylmorphine from MacFarlane Smith (Edinburgh, UK) and codeine, pholcodine, dihydrocodeine and morphine from Sigma (Poole, UK). Proprietary opiate preparations used, Panadeine (codeine), Pholcodine Linctus, Paramol (dihydrocodeine) and Collis Browne Mixture (morphine) were purchased from local pharmacies. The solvents pentane, dichloromethane and methanol were supplied by Rathburn (Walkerburn, UK) and diethylamine by Fisons (Loughborough, UK). Water was purified by distillation and subsequent treatment by a Milli-Q system (Millipore, Watford, UK). Bond Elut Certify (Analytichem, Harbor City, CA, USA) solid-phase extraction cartridges (300 mg) were used with a Vac-Elut ten-port vacuum manifold (Jones Chromatography, Hengoed, UK).

The HPLC system consisted of a Jasco PU980 pump coupled with a Jasco UV975 variable wavelength detector (Jasco, Tokyo, Japan) operated at 280 nm. Injection was performed with a Rheodyne 7125 (Cotati, CA, USA) six-port injection valve fitted with a 50-μl loop. Chromatographic columns (200×2 mm I.D.) were slurry packed in the laboratory at approximately 70 MPa with 3 μm Hypersil (HETP, Macclesfield, UK)

2.2. Pretreatment of urine samples

In the determination of opiates in urine hydrolysis pretreatment is usually incorporated to free opiates from mainly glucuronide conjugates depending upon the purpose of the assay. There are several hydrolysis procedures and, in the present work, urine samples were subjected to standard acid hydrolysis [15]. Concentrated hydrochloric acid (1 ml) was added to 5 ml of urine and the mixture vortex-mixed and then heated for 30 min at 120°C. After cooling the pH of the mixture was adjusted to between 7.0 and 8.0 using 10 *M* potassium hydroxide.

The extraction method used was that, based on solid-phase extraction, recommended for opiate analysis by GC [15]. After wetting of the cartridge with methanol (2 ml) and water (2 ml) a 5-ml urine or hydrolysed urine sample was applied and drawn slowly through the cartridge (2 min). After washing with water (2 ml), acetate buffer (pH 4) (1 ml) and methanol (2 ml) the analytes were eluted from the cartridge with 2 ml of a solvent of dichloromethane–isopropyl alcohol (80:20, v/v) which also contained 2% (v/v) ammonia. After reducing to dryness under nitrogen the analytes were reconstituted in 0.5 or 1.0 ml of dichloromethane–pentane (10:90, v/v). The hydrolysis procedure described

above may be omitted when detection of heroin or 6-monoacetylmorphine is being attempted. It may also be omitted when it is desired to determine free, i.e. unconjugated, opiate.

2.3. Chromatography

The mobile phase which gave optimum resolution among the analytes was prepared by adding 29.8 ml of dichloromethane to 65 ml of pentane and making up to 100 ml with methanol containing 0.5% (v/v) diethylamine. A volumetric flow-rate of 0.4 ml min^{-1} was used. All samples and standards were injected in dichloromethane–pentane (10:90, v/v). The composition of this injection solvent mixture was found to be critical in obtaining the separations reported and inferior resolution was obtained when any alternative injection solvent mixture was used.

2.4. Quantification and validation

Recovery from the urine matrix was determined by comparison of the peak heights obtained following the above pretreatment of urine samples ($n = 10$) spiked with the various opiates with those of standard solutions in the injection solvent of comparable concentration, taking into account the preconcentration inherent in the pretreatment. Urine samples were spiked with 50 ng ml^{-1} heroin and 6-monoacetylmorphine and 100 ng ml^{-1} of the remaining opiates. Linearity of peak-height ratio (analyte to internal standard) with concentration was determined by spiking urine with four concentrations of a mixture consisting of codeine, morphine, pholcodine, and dihydrocodeine over a range of 100–400 ng ml^{-1}. For heroin and 6-monoacetylmorphine the range of calibration standards was 50–200 ng ml^{-1} since these compounds are known to be present in appreciably lower concentrations in urine than morphine following heroin abuse [16]. A blank urine sample was included. Calibration of heroin and 6-monoacetylmorphine was carried out separately from the other opiates, because the hydrolysis step was omitted. The internal standard, nalorphine (50 ng ml^{-1}) was incorporated after hydrolysis and before extraction. Limits of detection were estimated by determining the signal-to-noise ratio for each opiate following extraction and chromatography of a sample containing 15 ng ml^{-1} of each opiate and the conventional detection limits calculated from this. The within-day and day-to-day precision were estimated by measuring the appropriate peak-height ratio for 10 replicate extractions at the concentration of 200 ng ml^{-1}.

No chromatographic interference was found from injections of 300 ng ml^{-1} solutions of the following drug compounds: aspirin, caffeine, chlordiazepoxide, dextropropoxyphene, diazepam, diphenylhydramine (before heroin), ephedrine (before codeine), hydrocodone (before pholcodine), lignocaine, naloxone, norcodeine, normorphine, papaverine, procaine, quinine, theobromine, theophylline.

The ability of the proposed method to detect and quantitate the commonly encountered opiates in vivo was validated in two ways. Firstly, a selection of urine samples, obtained as part of a pre-employment drug screening procedure, which had tested positive for opiate by EMIT and which subsequently were shown to contain specific opiates by GC–MS, were assayed by the proposed LC method. Secondly, urine samples from a single male volunteer were assayed for opiate by EMIT and the proposed method at different times, following ingestion of various proprietary drug preparations containing opiates.

3. Results and discussion

Fig. 1A shows a representative separation of the seven opiates dissolved directly in the injection solvent (concentration of heroin, 6-monoacetylmorphine, pholcodine and dihydrocodeine, 250 ng ml^{-1}; nalorphine, 375 ng ml^{-1}; codeine and morphine, 750 ng ml^{-1}). Separation is complete in approximately 16 min and resolution is greater than 2 between all pairs. Figs. 1B,C are chromatograms of blank urine and urine spiked with the opiates (concentration of heroin and nalorphine (internal standard), 50 ng ml^{-1}; 6-

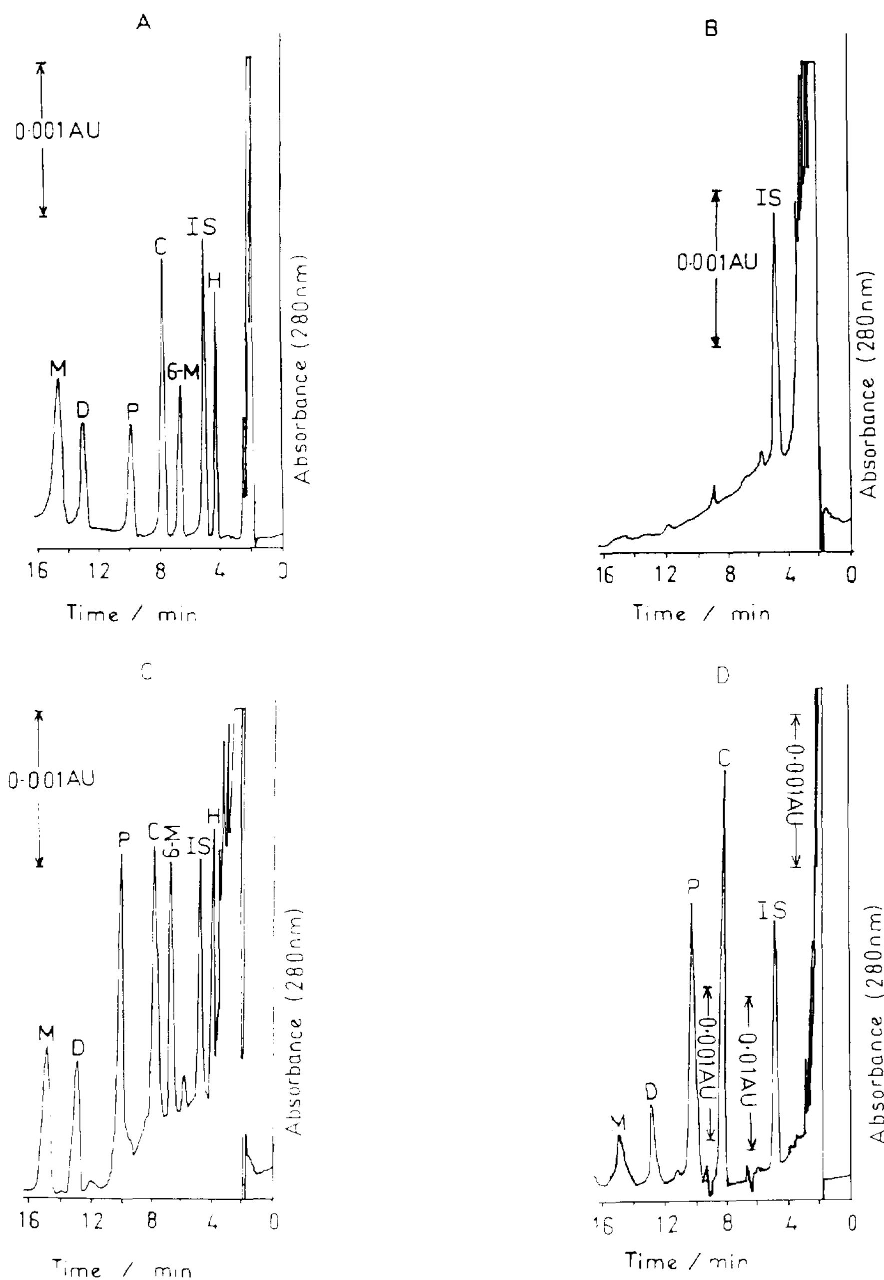

Fig. 1.(A) Chromatogram of a standard solution containing 250 ng ml^{-1} each of heroin (H), 6-monoacetylmorhine (6-M), pholcodine (P) and dihydrocodeine (D). 375 ng ml^{-1} each of nalorphine (internal standard, I.S.) and codeine (C) and 750 ng ml^{-1} of morphine (M). (B) Chromatogram of a hydrolysed and extracted blank urine containing 50 ng ml^{-1} nalorphine (internal standard, I.S.). (C) Chromatogram of a spiked urine sample after hydrolysis and extraction. Urine sample spiked with 50 ng ml^{-1} each of heroin (H) and nalorphine (internal standard, I.S.), 100 ng ml^{-1} each of 6-monoacetylmorphine (6-M), codeine (C) and dihydrocodeine (D) and 150 ng ml^{-1} of pholcodine (P) and morphine (M). (D) Chromatogram of an extracted hydrolysed urine sample from a male volunteer after ingestion at various times of codeine pholcodine and dihydrocodeine. It contains 1470 ng ml^{-1} codeine (C), 118.9 ng ml^{-1} pholcodine (P), 37.7 ng ml^{-1} dihydrocodeine (D) and 27.3 ng ml^{-1} morphine (M).

monoacetylmorphine, codeine and dihydrocodeine, 100 ng ml^{-1}; pholcodine and morphine, 150 ng ml^{-1}) after acid hydrolysis and subsequent sample pretreatment. While the hydrolysis procedure would not, in practice, be used for the determination of 6-monoacetylmorphine or free morphine or codeine the chromatograms represent the worst possible background obtained from endogenous urine components. In Fig. 1C it is seen that resolution is maintained and that heroin, the first eluting peak, can be measured in the presence of the endogenous components remaining after extraction. Fig. 1D shows a chromatogram obtained from a subject following ingestion of pholcodine, dihydrocodeine and codeine. As seen from Fig. 1D peaks for these opiates and also morphine formed by metabolism are evident.

Table 1 shows the main parameters for the quantitative validation of the method. Recoveries were greater than 79% and that for nalorphine, the internal standard, was 104.2 ± 5.4% (not shown in Table 1). The retention times relative to the internal standard shown in the table are an indication of the pattern of separation obtained. The relative standard deviation quoted is an overall value based on a six-month period. Within-day precision of retention is significantly higher. The correlation coefficients obtained for the regression lines of peak-height ratio on concentration are all greater than 0.990. The detection limit for the least sensitively detected compound, i.e. morphine, is 7ng ml^{-1} with a corresponding limit of quantification of 14 ng ml^{-1} which is well below the legal cut-off concentration for the EMIT detection of opiates of 300 ng ml^{-1}. The within-day precision for the method as a whole is seen to be in the region of 5% R.S.D.. The day-to-day precision is seen to be of the same magnitude.

Table 1
Analytical characteristics of the method for the various opiates

	Heroin	6-MAM	Codeine	Pholcodine	Dihydrocodeine	Morphine
Relative retention drug/I.S. (%R.S.D.) ($n = 4$)	0.785 (6.1)	1.25 (6.1)	1.51 (5.2)	1.99 (5.6)	2.57 (3.2)	3.04 (3.2)
Recovery (%) ($n = 10$)	89.1	82.7	82.0	88.4	79.7	79.3
Correlation coefficient	0.9987	0.9901	0.9900	0.9980	0.9909	0.9964
Slope of calibration line (± S.D.) $\cdot 10^3$	12.6 (0.26)	9.5 (0.51)	3.8 (0.32)	3.1 (0.11)	2.2 (0.17)	7.8 (0.04)
Signal-to-noise ratio at 15 ng ml^{-1}	20.2	11.0	14.0	9.6	10.6	4.0
Within-day precision at 200 ng ml^{-1} ($n = 10$) %R.S.D.	5.03	4.45	4.34	6.38	4.29	5.85
Day-to-day precision at 200 ng ml^{-1} ($n = 10$) %R.S.D.	5.43	4.92	5.11	7.22	6.1	8.07

The resolution and peak capacity demonstrated in the present work are the result of the high efficiency of the 3-μm stationary phase coupled with the selective solvent mixture developed, in which the concentration of diethylamine was found to be critical. In addition it was found experimentally that concentrations of dichloromethane in the injection solvent in excess of 20% (v/v) resulted in a marked loss of efficiency and resolution. This is assumed to be analogous to the peak compression effects [17,18] which are well established in reversed-phase systems. Use of a 2-mm I.D. column provided the anticipated increase in mass sensitivity which in turn allowed the wavelength of detection to be increased from the 220–230 nm often employed [11] for the UV detection of the opiates to the secondary maximum at 280 nm. This resulted in lower baseline noise and a very much reduced solvent front due to higher selectivity in the presence of endogenous co-extractions.

Table 2 shows the drugs detected in ten representative urine samples obtained from different subjects during pre-employment drug screening for opiate abuse by EMIT, by GC–MS (in different commercial laboratories) and by the present method. All the samples shown tested positive for opiate by EMIT. The GC–MS and the present LC-UV methods show good correspondence with respect to the individual opiates detected although quantitative information on the concentration of each was not supplied from

Table 2
Comparison of opiate detection and determination in the urine of 10 different human subjects by EMIT, GC–MS and the LC-UV method

Sample	EMIT result Calibrator 300 ng ml^{-1}	GC–MS		HPLC	
		Centre	Drug identified	Drug identified	Concentration (ng ml^{-1})
1	Positive	A	Codeine	Codeine	324.6
			Morphine	Morphine	93.2
2	Positive	A	Morphine	Dihydrocodeine	42.4
				Morphine	97.4
				Pholcodine	842.1
3	Positive	B	Codeine	Codeine	337.1
				Morphine	78.2
4	Positive	A	Morphine	Morphine	115.3
5	Positive	B	Pholcodine	Pholcodine	320.7
				Dihydrocodeine	77.5
				Morphine	27.1
6	Positive	A	Pholcodine	Pholcodine	652.8
				Morphine	45.5
7	Positive	B	Codeine	Codeine	531.6
				Morphine	136.3
8	Positive	A	Codeine	Codeine	843.2
				Morphine	75.3
9	Positive	B	Morphine	Dihydrocodeine	162.1
				Morphine	234.8
10	Positive	C	Dihydrocodeine	Dihydrocodeine	9870.3
			Morphine	Morphine	402.9

Table 3
Results of opiate determinations by EMIT and LC-UV method on a single volunteer at various times following ingestion of single and multiple opiates

Time since first dose/(h)	Preparation ingested	Drug (dose)	EMIT results (calibrator, 300 ng ml^{-1})	HPLC	
				Drug found	Concentration (ng ml^{-1})
Dihydrocodeine					
0	Paramol	Dihydrocodeine tartrate BP. (15 mg)	Negative	Dihydrocodeine	–
				Morphine	–
1		Paracetamol BP. (500 mg)	Positive	Dihydrocodeine	10204.0
				Morphine	22.4
3			Positive	Dihydrocodeine	3571.2
				Morphine	15.4
5			Positive	Dihydrocodeine	4517.3
				Morphine	8.1
8			Positive	Dihydrocodeine	1824.1
				Morphine	5.9
24			Positive	Dihydrocodeine	880.1
				Morphine	5.4
Pholcodine					
0	Pholcodine Linctus BP.	Pholcodine BP. (10 mg)	Negative	Pholcodine	–
				Morphine	–
1			Positive	Pholcodine	1960.3
				Morphine	60.1
3			Positive	Pholcodine	3200.4
				Morphine	47.8
5			Positive	Pholcodine	2620.3
				Morphine	67.4
8			Positive	Pholcodine	1340.2
				Morphine	70.4
24			Positive	Pholcodine	668.6
				Morphine	103.8
Codeine after previous ingestion of dihydrocodeine and pholcodine					
−120	Paramol	Dihydrocodeine tartrate BP. (15 mg) Paracetamol BP. (500 mg)			
−96	Pholcodine linctus BP.	Pholcodine BP. (10 mg)			
0	Panadeine Co.	Codeine (16 mg) Paracetamol BP. (500 mg)	Positive	Dihydrocodeine	113.8
				Pholcodine	304.8
				Codeine	–
				Morphine	67.8
1			Positive	Dihydrocodeine	47.6
				Pholcodine	199.5
				Codeine	3190.1
				Morphine	60.4
3			Positive	Dihydrocodeine	37.7
				Pholcodine	118.9
				Codeine	1470.3
				Morphine	27.3

Table 3 (*continued*)

Time since first dose/(h)	Preparation ingested	Drug (dose)	EMIT results (calibrator, 300 ng ml^{-1})	HPLC	
				Drug found	Concentration (ng ml^{-1})
5			Positive	Dihydrocodeine	34.4
				Pholcodine	388.9
				Codeine	2050.9
				Morphine	43.1
24			Negative	Dihydrocodeine	24.4
				Pholcodine	87.3
				Codeine	12.4
				Morphine	–
Morphine after previous ingestion of pholcodine					
−120	Pholcodine Linctus BP.	Pholcodine BP. (10 mg)			
0	Collis Browne Mixture.	Morphine (3 mg)	Positive	Pholcodine	221.5
				Morphine	122.5
1			Positive	Pholcodine	104.1
				Morphine	754.5
3			Positive	Pholcodine	51.2
				Morphine	575.3
5			Positive	Pholcodine	118.8
				Morphine	439.3
8			Positive	Pholcodine	68.0
				Morphine	176.7
24			Positive	Pholcodine	47.2
				Morphine	73.1

commercial laboratories as routine. In determining the identity of the drug consumed when a morphine peak is detected in urine, two criteria must be fulfilled before consumption of morphine is presumed. The level of morphine must be above 200 ng ml^{-1} and the ratio of legal drug to morphine must be less than 0.5 [19,20]. The data in Table 2 show that the method is capable of providing the information required, i.e. the absolute level of morphine and the legal drug to morphine ratio. For all of the samples shown in Table 2 the presumption would be consumption of legal drug.

Table 3 shows the results obtained by EMIT testing and by the present LC method at different times for a single volunteer following ingestion of various preparations containing the generally available legal opiates. Results obtained following a single ingestion of dihydrocodeine are shown. It can be seen that both dihydrocodeine and morphine can be detected by the LC method between 1 and 24 h. Neither drug was detected at baseline. Next the results of a similar procedure following a single ingestion of 10 mg of pholcodine are presented. The results parallel those shown for dihydrocodeine. They also show that the ingested drug to morphine ratio and the total morphine level can be determined. The 3rd part of Table 3 reports the concentration of drugs found when a single volunteer ingested several legal opiates; 120 h before the start of measurements the subject consumed 15 mg of dihydrocodeine and 96 h before the start of measurements 10 mg of pholcodine. At time zero 16 mg of codeine were consumed. The drug concentrations reported show that it was possible to determine the concentration of all drugs consumed and morphine produced by metabolism, over a 24-h period. The last part of Table 3 reports the urine drug levels found when in a

separate experiment the subject consumed 10 mg of pholcodine 96 h before starting measurements and 3 mg of morphine at time zero. The results show that the proposed method is capable of detecting both drugs and simulates the condition where morphine consumption could be presumed; e.g. at 3 h the morphine concentration is greater than 200 ng ml^{-1} and the pholcodine to morphine ratio is 0.09.

Table 3 also shows complete correspondence between the LC results and the non-specific EMIT test for opiates, the negative EMIT test for the 24-h sample in the 3rd part of Table 3 arises from the levels of opiates being below those required for reliable EMIT detection.

The above results show that the proposed method based on HPLC with direct ultraviolet detection at 280 nm is capable of quantifying the commonly encountered opiates used medicinally in the presence of heroin and 6-monoacetylmorphine in both spiked urine samples and in urine samples from human subjects. It has the capability of providing data to assess the probability that a positive EMIT opiate result is the consequence of consumption of one or more legally used opiates by allowing determination of the appropriate morphine to legal opiate ratio and may thus obviate the need for GC–MS confirmation.

References

[1] V. Nitsche and H. Mascher, *J. Pharm. Sci.*, 73 (1984) 1556.

[2] J. Svensson, A. Rane, J. Sawe and F. Sjoquist, *J. Chromatogr.*, 230 (1982) 427.

[3] G. Chari, A. Gulati, R. Bhat and I.R. Tebbett, *J. Chromatogr.*, 571 (1991) 263.

[4] I.R. Tebbett, *Chromatographia*, 23 (1987) 377.

[5] I.S. Lurie, S.M. Sottolano and S. Blasof, *J. Forensic Sci.*, 27 (1982) 519.

[6] G.K. Poochikian and J.C. Craddock, *J.Chromatogr.*, 171 (1979) 371.

[7] W.M. Posey and S.N. Kimble, *J. Anal. Toxicol.*, 8 (1984) 68.

[8] M. Johansen, F. Tonnesen and K.E. Rasmussen, *J. Chromatogr.*, 572 (1992) 283.

[9] S.K. Soni and S.M. Dugar, *J. Forensic Sci.*, 24 (1979) 437.

[10] A.T. Andesen, M. Krogh and K.E. Rasmussen, *J. Chromatogr.*, 582 (1992) 123.

[11] D. Weilbo, R. Bhat, G. Chari, D. Vidyasagar, I.R. Tebbett and A. Gulati, *J Chromatogr.*, 615 (1993) 164.

[12] J.G. Umans, T.S.K. Chiu, R.A. Lipman, M.F. Schultz, S. Shin and C.E. Inturrisi, *J. Chromatogr.*, 233 (1982) 213.

[13] R.F. Venn and A. Michalkiewicz, *J. Chromatogr.*, 525 (1990) 379.

[14] S.D. Ferrara, L. Tedeschi, G. Frison and F. Castagna, *J. Anal. Toxicol.*, 16 (1992) 217.

[15] Opiate extraction procedure, Bond Elut Certify, Instruction Manual, Analytichem International, Division of Varian, 1989.

[16] Report of the Substance Abuse Testing Committee, *Clin. Chem.*, 34 (1990) 605.

[17] K. Slais, D. Kourilova and M. Krejci, *J. Chromatogr.*, 282 (1983) 383.

[18] R.B. Taylor, K.E. Kendle, R.G. Reid and C.T. Hung, *J. Chromatogr.*, 385 (1987) 383.

[19] M.C. Dutt and K.L. Tong, in T.A. Gough (Editor), *The Analysis of Drugs of Abuse*, John Wiley and Sons Chichester, 1991, Ch. 11, pp. 416–419.

[20] M.A. ElSohley and A.B. Jones, *Forensic Sci. Rev.*, 1 (1989) 14.