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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MORPHINE IN BIOLOGICAL SAMPLES: AN OVERVIEW OF SEPARATION METHODS AND DETECTION TECHNIQUES

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SUMMARY

High-performance liquid chromatography with electrochemical detection at present suits most of the needs of toxicologists for the determination of morphine and some related compounds in biological samples, although fluorescence detection is still a useful alternative. Chemiluminescence detection may be promising, but needs further optimization of its coupling with HPLC to give the best performances. Morphine detection by absorbance spectrophotometry does not seem to allow the degree of sensitivity and selectivity from matrix interferences that is required in most instances. However, this approach is useful when morphine congeners undetectable by alternative means (i.e., heroin and morphine-3-glucuronide) are to be determined or when a general toxicological screening is required.

INTRODUCTION

Morphine was first isolated by Serturner in 1803 and since then a number of related compounds have been synthesized in order to overcome the main drawbacks of the parent drug, i.e., tolerance and dependence, including in 1898 heroin, the 3,6-diacetyl ester of morphine, which subsequently became one of the main drugs of abuse in the western societies.

In a variety of human and animal tissues heroin undergoes rapid enzymatic

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deacylation to 6-acetylmorphine, mainly catalysed by blood esterases. 6-Acetylmorphine is converted to morphine, presumably in the liver. Morphine is probably the very active principle of heroin, as heroin itself and 6-acetylmorphine have very little affinity for the opiate receptors in brain tissue [1].

Morphine is extensively metabolized in both the liver and intestine to the 3-glucuronide, which is the main excretion form, and in small amounts (0.3% of an administered parenteral dose) [2] to morphine-6-glucuronide. Normorphine is produced by N-demethylation although in limited amounts (5% of an oral dose of morphine) [3]. Other minor metabolites, including morphine-3,6-diglucuronide and codeine, have been reported [4]. Morphine-3-glucuronide is pharmacologically inactive [5]; in contrast, morphine-6-glucuronide has been reported to exert analgesic activity [2].

On this basis it is clear that the purposes of morphine analysis in biological samples are two-fold: on the one hand, monitoring therapeutic levels in patients and drug concentrations in human and animal pharmacokinetic studies, and on the other, investigating heroin abuse for epidemiological purposes or drug abuse control, or the causes of intoxication or death in cases of clinical, pathological or forensic interest.

Unconjugated morphine is the analyte of interest when pharmacological or toxic effects are to be correlated with morphine or heroin administration. Its concentrations in plasma from subjects on morphine therapy are roughly in the range 8–80 ng/ml [2]. Mainly because of tolerance development, lethal levels have not yet been clarified, but could tentatively be considered to be as low as 50 ng/ml [1].

The presence of morphine glucuronides in blood and urine is roughly related to a fairly recent opioid intake, and is commonly investigated for screening purposes. In these cases urine is the biological fluid that almost always is preferred. Urine 'total' morphine (i.e., free plus conjugated) levels can range from micrograms per millilitre during heavy and chronic heroin intake down to nanograms per millilitre after only a few days of abstinence.

An alternative way of investigating opioid abuse histories is to determine morphine in hair. The drug passes from the blood into the hair roots and there is slowly embedded into the hair matrix, where it remains throughout lifetime of the hair [6].

In recent years, mainly because of laboratory controls on subjects undergoing drug detoxication programmes, morphine determination in biological samples has become nearly a routine procedure in many laboratories involved in clinical and forensic toxicology. Demand for controls on drug abuse in applicants for jobs involving a particular responsibility toward public health is also growing.

ANALYTICAL APPROACHES

Several techniques have been proposed for determining morphine and related compounds in biological fluids and tissues with the required degree of sensitivity, specificity and reliability. As UV spectroscopy clearly lacks sensitivity and spec-

ificity, spectrofluorimetric assays have been proposed [7–9], but they have been reported to be of poor specificity [10,11].

At present, most laboratories adopt immunological assays, as they can be carried out directly on unextracted samples. Radioimmunoassays (RIA) [12] allow picogram amounts of morphine to be detected, but require authorized personnel and areas. Enzyme immunoassay (EIA) (EMIT®, Syva) and fluorescence polarization immunoassays (FPIA) (TDX®, Abbott) are fully automated and, although less sensitive, do not involve handling of radioactive materials. Also, a qualitative method based on haemoagglutino inhibition (Drug Test EM, Boehringer-Biochemia) is commercially available. However, immunoassays are impaired by possible cross-reactions of antisera with other opiates [13] and by non-specific interferences (pH, ionic strength). This led to the recommendation of the Toxicological Section of the American Academy of Forensic Sciences of confirming results from immunological methods by alternative techniques based on different analytical principles, but with comparable sensitivity [14].

Among the chromatographic methods in common use, thin-layer chromatography (TLC) is simple and inexpensive and, owing to the possibility of managing large routines, is widely used for urine screening. However, it suffers from a certain lack of sensitivity and specificity, even if derivatization procedures [15,16] can improve its performance. In addition, quantitation is less easy and reliable than in column chromatography.

Much better specificity and reliability are inherent in gas chromatography (GC), but only mass fragmentography [17] and electron-capture detection [18,19] achieve sensitivity limits comparable to those of the best RIAs. GC methods need careful and time-consuming sample preparation, including derivatization to reduce the high polarity of morphine and to make electron-capture detection possible. For these reasons, high-performance liquid chromatography (HPLC) is being adopted in an increasing number of laboratories, because of its inherent features of specificity, reliability, sensitivity and, to a certain extent, reduced needs for sample preparation.

GENERAL CONSIDERATIONS ON THE HPLC OF MORPHINE

HPLC methods for the determination of morphine in biological fluids can rely on a large background concerning the chromatography of basic drugs and illicit heroin. Since the early 1970s, various successful HPLC applications have been reported in this field. Methods using ion exchange [20], reversed-phase [21] or reversed-phase ion-pair separations [22] or chromatography on unmodified silica with aqueous methanol [23] or non-aqueous eluents [24,25], sometimes with counter ions added [26], have been developed.

The use of silica columns with polar eluents (aqueous methanol with ammonia or ammonium salts added), first introduced by Jane in 1975 [23], has been adopted in many forensic laboratories, mainly because of its excellent resolving power for a wide range of drugs, including morphine and other opioids [27]. Although the mechanisms of separation are not yet fully clarified, evidence is accumulating in favour of cation exchange [28]. Further studies have shown that silica columns

provide fairly good separations also with non-aqueous, primarily methanolic, eluents, modified by ionic compounds dissociated in organic media [24,25].

Despite the need for basic eluents, it has been claimed that, under these conditions, deterioration of silica columns is minimal [23,24]. Nevertheless, serious problems of reproducibility among different batches of silica, even from the same producer, still remain [27,29]. In addition, the influence on column performance of polar impurities present in the samples and of the water content of the mobile phase [30] are critical with silica packings. The difficulty of regenerating silica columns must also be taken into account. Finally, promising separations of alkaloids, including morphine, have been achieved with alumina [31] and poly(styrene-divinylbenzene) polymer packings [32].

Other workers have proposed the use of reversed-phase chromatography, which seems to require less stringent analytical conditions. Either C_{18} [21] or amino- [33] or cyano-bonded [34] phases have been used with good results for most illicit heroin components, but tailing of the morphine peak has often been reported. Fairly good column efficiency and peak shape for basic drugs have been reported by using reversed-phase ion-pair chromatographic systems [22], but some problems with the resolution of opiate drugs still remain.

All of these studies have been focused on the separation of a number of drugs and/or heroin additives and adulterants, but were not tailored to the purposes of analysing biological fluids, where resolution from matrix components is often the main problem. Moreover, these methods generally do not allow high sensitivity, but stress the identification power of UV detectors [35,36].

On the other hand, as sensitivity is of major concern when HPLC methods are applied in the biomedical field, we shall next review the different HPLC strategies for morphine determination classified according to the detection techniques, on which sensitivity mainly relies.

Although electrochemical detectors are by far the most widely used, UV detectors have been chosen by some workers, as they often are basic components of HPLC systems and theoretically allow the detection of many other drugs of toxicological interest.

CHROMATOGRAPHIC PROCEDURES WITH UV-VIS DETECTION

The UV spectrum of morphine has a typical absorbance maximum at 285 nm, but its molar absorptivity is poor. Therefore, when sensitivity has to be stressed, most workers prefer to operate at 210–220 nm. However, in this wavelength range the detector selectivity is poor and sample preparation becomes critical. The following set-ups of chromatographic systems may serve as typical examples of exploiting UV–VIS detection for the analysis of morphine and related compounds.

The first attempt to use reversed-phase HPLC on C_{18} silica with UV detection was published by Ulrich and Ruegsegger [37]. Sensitivity was improved by Posey and Kimble [38] by using reversed-phase HPLC with UV detection at 210 nm. The method allows the simultaneous determination of morphine and codeine in urine and blood, but requires a complex sample preparation.

Blood or urine samples spiked with nalorphine (internal standard, I.S.), pre-

viously adjusted to pH 9.2, were extracted with chloroform–isopropanol–heptane (50:17:33) and then back-extracted with 0.2 mol/l hydrochloric acid. The aqueous phase was then washed with heptane, made alkaline and re-extracted with chloroform. Finally, the organic phase was evaporated and the residue reconstituted in 100 μl of mobile phase; 10 μl were injected into the chromatograph. The separation was carried out on a CN-bonded column with a mobile phase consisting of methanol–0.1 mol/l phosphate buffer (40:60) at a pH of 6.8. The column effluent was monitored at 210 nm. Under these conditions, the peaks of morphine, codeine and I.S. were well resolved from each other and from those of a number of opiate and non-opiate compounds. The linearity of the assay was within the range 50–500 ng/ml; the limit of quantitation was 50 ng/ml. Later, the same workers [39] applied the method to the determination of norcodeine, in addition to morphine and codeine, in urine of subjects taking codeine.

Another application of HPLC–UV methods concerns the simultaneous determination of morphine and its glucuronides. For this purpose, Svensson et al. [40] used a reversed-phase ion-pair system with detection at 210 nm. For sample preparation, disposable cartridges packed with coarse C_{18} silica (Sep-Pak C_{18}, Waters Assoc.) were used. Two purification steps through Sep-Pak C_{18} were required to obtain an extract suitable for chromatography. The extraction recovery was about 90% for both morphine and morphine-3-glucuronide. The use of an ion-pairing agent (dodecyl sulphate) was required in order to obtain a suitable retention time of morphine even at pH 2.1, necessary to separate simultaneously morphine glucuronides, by suppressing the ionization of the glucuronic acid group ($pK_a = 3.2$). The limit of detection was 5 ng/ml for both morphine and morphine-3-glucuronide. In urine from morphine-treated patients, in addition to morphine, normorphine and morphine-3-glucuronide, an unknown peak was found, which on the basis of the UV spectrum and of its ability to be hydrolysed by β-glucuronidase, was identified as morphine-6-glucuronide.

Taking advantage of the sensitivity and low selectivity of UV detectors at short wavelengths, Umans et al. [41] devised a method for the simultaneous determination of heroin, 6-acetylmorphine and morphine in biological fluids. In order to avoid enzymatic and spontaneous hydrolysis of the acetylated compounds, rapid freezing of samples was applied, followed by a very complex liquid–liquid extraction in mild alkaline conditions. Chromatographic separation was carried out on an underivatized silica column. The mobile phase was acetonitrile–methanol (75:25), buffered to pH 7 with ammonium acetate, to avoid heroin breakdown; detection was achieved by measurement of the UV absorbance at 218 nm, which was a compromise between maximum sensitivity and acceptable noise. The limit of detection was 12.5 ng/ml.

Because of its short half-life in vivo and its intrinsic instability, the determination of heroin in biological fluids is interesting only for pharmacokinetic studies. 6-Monoacetylmorphine is a more useful marker of recent heroin intake [42], but unfortunately its levels are below the detection limit of HPLC–UV methods.

Recently, an HPLC method using an ODS column with residual silanol groups and UV detection at 214 nm was reported for the determination of morphinone in urine and bile of guinea pigs [43]. This compound is suspected to be a toxic

metabolite of morphine. Because of its instability, morphinone was determined as its 2-mercaptoethanol adduct.

Derivatization with dabsyl chloride has been reported as a means of simplifying sample preparation and, to a certain extent, improving sensitivity. Dabsyl chloride, first synthesized by Lin and Chang in 1975 [44], on reaction with morphine gives a fairly stable product with an orange colour; the molar absorptivity of dabsylmorphine, at its absorption maximum of 450 nm, is about 30 times higher than that of morphine at 280 nm. This prompted the development of both TLC and HPLC methods for the determination of morphine in biological fluids [16]. Detection in the visible region suffers from many fewer problems of interferences from the matrix than detection in the low UV region. Therefore, sample preparation is less demanding and, in fact, has been limited to a one-step extraction of urine, previously adjusted to pH 9.1, with chloroform–isopropyl alcohol (9:1). In addition, dabsyl derivatization and extraction of the derivative with toluene were required. Normal-phase chromatography on a silica column can be used in this instance. Detection at 436 nm was carried out with a deuterium source, operating out of the optimum range of the detector. Under these conditions the limit of detection was 75 ng/ml.

CHROMATOGRAPHIC SYSTEMS WITH FLUORESCENCE DETECTION

An alternative method of detection is fluorimetry. Morphine shows weak native fluorescence, but under alkaline conditions can be oxidized to a highly fluorescent dimer, pseudomorphine. This reaction, common to other morphine congeners, requires, according to Darwin and Cone [45], three structural features: the presence of a free hydroxy group at the C-3 position, the absence of a carbonyl group at the C-6 position and the presence of a furan oxygen bridge.

Dimerization to pseudomorphine with fluorescence detection was proposed for the direct determination of morphine in body fluids [8,9], but proved to be nonspecific and too dependent on matrix composition to provide reliable results.

Next we shall summarize typical examples of the use of fluorescence detection in chromatographic separations of morphine.

Conversion of morphine to a fluorescent product (pseudomorphine) was reported by Jane and Taylor [46]; oxidation was performed on-column with potassium hexacyanoferrate(III). Urine was extracted with chloroform–isopropyl alcohol (9:1). Dihydromorphine (I.S.) reacts in an analogous way to morphine and therefore when both morphine and dihydromorphine are present, three fluorescent products are formed, the two dimers and a mixed dimer. Chromatographic separation was carried out on a silica column with methanol–2 mol/l ammonia solution–1 mol/l ammonium nitrate solution (30:20:10). The fluorimetric detector was operated at 320 nm (excitation) and 436 nm (emission). Under these conditions, morphine dimer, morphine–dihydromorphine mixed dimer and dihydromorphine dimer were eluted in that order. The detection limit was 4 ng of morphine injected and quantitation in urine was possible at levels from 100 ng/ml to 10 μg/ml. In spite of some problems with sensitivity, probably owing to

incomplete dimerization, this method first allowed the use of HPLC for the determination of morphine in biological fluids.

Post-column morphine dimerization to pseudomorphine was proposed by Nelson et al. [47]. This approach was claimed to be more reproducible and reliable than pre-column reactions, mainly because no artefact formation can occur and there is no need for completion of reaction.

According to Nelson [48], morphine was separated in the original form in a reversed-phase system on an ODS silica column with a mobile phase consisting of methanol–0.1 mol/l aqueous potassium bromide (12.5:87.5) adjusted to pH 3 with phosphoric acid. The derivatizing reagent, consisting of 50 mg of potassium hexacyanoferrate(III) in 250 ml of 4 mol/l ammonia solution, was added post-column by an auxiliary pump into a 5 m×0.3 mm I.D. reaction coil connected to a filter fluorimeter. Urine samples were extracted with chloroform–isopropyl alcohol according to Jane and Taylor [46]. Blood samples required an additional back-extraction from the organic mixture with 0.1 mol/l sulphuric acid and a re-extraction with the solvent mixture. The detection limit was 10 ng of morphine injected.

Although theoretically interesting, post-column derivatization to pseudomorphine suffers from major problems of sensitivity, which were attributed to the combined effect of incomplete derivatization and quenching of the fluorescent response (or pseudomorphine breakdown, according to Jane and Taylor [46] by hexacyanoferrate(III) . In practice they found that only 20% of the optimum theoretical response was achieved. In addition, a dilution effect due to the post-column addition of the reagent has to be taken into account.

Later, Nelson [48] reported that, if the dimerization reaction was carried out in micellar solution, on addition of a non-ionic surfactant (Triton X-100) a significant increase in the fluorescence response was observed. This phenomenon was attributed to a kind of micellar catalysis of the phenoxy free radical coupling to give dimers and to the protection of the product from further oxidation. Unfortunately, the fluorescence gain for morphine was only 25%.

The phenolic oxidative coupling of morphine and related opiates has also been adopted for the determination of 6-acetylmorphine in urine. This heroin metabolite, successively converted to morphine, is per se interesting when a recent intake of heroin has to be investigated [42]. Moreover, it is the only marker of heroin use in addicts undergoing treatment with morphine. As 6-acetylmorphine is present in biological fluids at levels of a few nanograms per millilitre, sensitivity is a critical point. Derks and co-workers [49,50] reported an HPLC method using pre-column oxidation of 6-acetylmorphine to acetylmorphine–morphine fluorescent dimers in the presence of a purposely added excess of morphine. This choice was related to the need to avoid the cross-production of too many mixed dimers from the different opioid metabolites present in urine at low levels. As the determination of morphine was of no interest, addition of morphine in a large excess made coupling reactions in which no morphine was involved highly unlikely. Under these conditions the main products were the morphine dimer and mixed dimers of morphine with each of the minor opioid metabolites, including 6-acetylmorphine. It was claimed that the sensitivity with a post-column reaction system according to Nelson et al. [47] was poor.

Two Extrelut (Merck) columns and back-extraction with 50 mmol/l sulphuric acid were used for urine. The extracts were oxidized with 0.015 mol/l potassium hexacyanoferrate(III) in Tris buffer (pH 8.5); the reaction was stopped and the mixture directly injected. Either a silica column with aqueous acetonitrile as eluent or a reversed-phase ODS system were used, with comparable results, but as the former eluent caused excessive wear of the piston seal of the high-pressure pump, the reversed-phase system was preferred. The fluorescence detector was operated according to Jane and Taylor [46]. The limit of detection in urine was 1 ng/ml.

Pursuing a simpler way of obtaining a highly fluorescent morphine derivative, based on previous work by Frei et al. [51], we used dansyl chloride for the determination of morphine in human serum and urine [52]. A highly fluorescent and stable product was obtained by reaction of dansyl chloride with morphine at basic pH. However, because of the poor specificity of the reaction, when sensitivity had to be stressed, such as in plasma samples, a complex extraction procedure, according to Felby et al. [53], had to be adopted. Briefly, extraction of alkalinized plasma with chloroform–isopropyl alcohol (4:1) was followed by washing of the organic phase with borate buffer, back-extraction with 1 mol/l sulphuric acid and re-extraction with the same organic mixture. The organic phase was finally evaporated. In contrast, urine samples could be simply extracted with a ready-to-use commercial kit, Toxi-Tubes A (Analytical Systems). The residues from both procedures were reacted with 100 μl of dansyl chloride in acetone in the presence of 0.2 mol/l sodium carbonate solution. After incubation for 3 h at room temperature, the derivatives were extracted with 1 ml of toluene, followed by evaporation to dryness. The residues were dissolved in the mobile phase and injected. Because of the quenching effect on dansyl fluorescence caused by polar solvents, normal-phase chromatography was chosen, using a silica column with hexane–isopropyl alcohol–ammonia solution (97:3:0.3) as the eluent. The detector was a filter fluorimeter with excitation in the wavelength range 330–380 nm and emission in the range 410–500 nm. Under these conditions, using the most complex extraction procedure, fairly clean chromatograms were obtained, even with whole blood, at levels down to 10 ng/ml. The simplified extraction allowed us to measure morphine levels in urine, where the alkaloid is present at higher levels. This method was also adopted by our group to investigate morphine in hair as a marker of chronic opiate abuse [54].

Micellar chromatography with detection of the native fluorescence of morphine has been reported as a means of allowing direct injection of untreated serum or urine [55]. The micellar mobile phase used consisted of 0.03 mol/l sodium dodecyl sulphate (SDS) in distilled water containing 10% *n*-propanol; the column was 10-μm μBondapak C_{18} (Waters Assoc.). A fluorimetric detector with excitation at 215 nm (deuterium lamp) and a 300 nm emission cut-off filter gave a sensitivity of 300 ng/ml. Under these conditions 20 μl of untreated serum or urine were sufficient for analysis. No problems with protein precipitation or column clogging were observed, but drug elution occurred on the tail of a large front of endogenous compounds, which prevented the use of the most sensitive detector ranges. Although the reported limit of detection is only 300 ng/ml, this is comparable to that of enzyme immunoassays. Although this seems a promising ap-

proach, especially in clinical toxicology, we point out that no data on possible interferences by other drugs are available at present.

Direct injection of urine or diluted serum samples onto an ODS silica column has been reported by Nelson et al. [56]. They used a reversed-phase system, incorporating bromide ions, with off-line determination of the collected fractions of the eluate by enzyme immunoassay or radioimmunoassay. This method was mainly intended as a means of identifying compounds that cross-react in commercial morphine immunoassays.

CHROMATOGRAPHIC SYSTEMS WITH CHEMILUMINESCENCE DETECTION

Oxidations are frequently sources of chemiluminescence (CL), as they involve large free energy changes. In particular, as many conditions that influence the conversion of morphine to fluorescent dimers also affect the production of CL, dimerization to pseudomorphine has been hypothesized to be at the origin of the CL of morphine. It has also been reported that the CL of morphine strongly depends on the use of permanganate as the oxidizing agent, whereas other oxidants used for pseudomorphine production are ineffective. In addition an acidic medium is required, and 'polyphosphoric' (mainly tetraphosphoric) acid gives the best response. It was proposed [57] that a manganese species, formed when permanganate is reduced by morphine, forms a complex with the polyphosphoric acid, and that this complex is the emitter.

In particular, CL seems to be a promising tool for investigating biological fluids lacking almost entirely any background emission and interferences from endogenous compounds. As a number of morphine congeners give CL, coupling with HPLC has been proposed for achieving the required specificity [58]. The following setups can be used for this purpose.

A suitable eluent for CL detection consisted of 0.01 mol/l polyphosphoric acid–methanol (87.5:12.5) at pH 2.2. A pH-resistant polymeric [poly(styrene–divinylbenzene)] column was used. Methanol was used as the organic modifier of the mobile phase, as it proved to have only a weak quenching effect on CL. The oxidant solution (0.6 mmol/l potassium permanganate) was mixed with the eluent from the column through a T-piece. An original flow-through CL detector was placed downstream. Although claimed to be very selective, this HPLC–CL method required complex sample preparation. Urine was extracted by a solid–liquid procedure [40], slightly modified to suit the requirements of CL. The simpler liquid–liquid method [46] was less efficient at removing impurities. According to Abbott et al. [58], solid–liquid extraction is preferable to the liquid–liquid method also for whole blood. Under these conditions, the limit of detection was 50 ng/ml in biological fluids and 25 ng/ml in aqueous solution (2.5 ng on-column). This seems poor compared with the detection limit of 0.7 pg per injection using flow injection, as reported by the same workers [57]. Nevertheless, the described HPLC–CL method had to cope with contrasting requirements of liquid chromatography and CL detection.

CHROMATOGRAPHIC SYSTEMS WITH ELECTROCHEMICAL DETECTION

After Kissinger et al. [59] had demonstrated the feasibility and perspectives of electrochemical detection (ED) in HPLC, White [60] was the first to report an HPLC determination of morphine using electrochemical oxidation. Because of its sensitivity and, to a certain extent, specificity, this technique has now become the most popular for the determination of morphine in biological samples. Although still controversial [61], it seems likely that in morphine one-electron oxidation occurs at the 3-hydroxy group, previously ionized, followed by dimerization of the free radical to pseudomorphine. A two-electron oxidation of morphine and/or further oxidation of pseudomorphine have also been hypothesized [60,62]. Fluorescence determination of morphine via electrogenerated pseudomorphine was reported by McLeod and West [63], although, to the best of our knowledge, it has been not yet been applied to HPLC. Since White's first work [60], a number of papers have appeared, reporting major or minor changes to obtain either higher sensitivity or lower interferences from matrix components or the possibility of detecting a wider range of drugs. Let us now exemplify the applicability of ED in morphine determination.

As far as chromatographic separation is concerned, underivatized silica with aqueous methanol–acetonitrile [60,64,65] or non-aqueous ionic eluents [66] at basic pH has been used. However, in most instances reversed-phase [67–75] or reversed-phase ion-pair systems [76–81] on C_{18} bonded phases [67–74,76–79,81] were used; the use of cyano- [80] or phenyl-bonded [75] silica columns has also been reported. Generally, acidic mobile phases with the usual organic modifiers have been employed, together with various ion-pairing agents. In a single instance [72] the eluent was methanol–water made alkaline with 0.1% ammonia solution.

Most workers have used amperometric detectors with single glassy carbon electrodes [60,64–78,81] operated at potentials ranging from +600 to +1000 mV vs. Ag/AgCl or SCE reference electrodes. Higher voltages (1100–1200 mV) were adopted when codeine also had to be detected [64,75]. The choice of the electrode potential was made with the aim of maximizing the signal-to-noise ratio under the actual working conditions. The limit of detection was excellent, being in the range 1–2 ng/ml.

Most workers dealing with biological fluids (serum, plasma, blood) [60,67,70,72,74,81] reported complex procedures in the liquid phase, consisting in a first extraction of the sample, previously buffered at pH ca. 9, with various organic mixtures, back-extraction with dilute acids, alkalinization and re-extraction with an organic phase, which was finally evaporated. Other workers omitted the organic re-extraction and directly injected the back-extracted sample in the acidic aqueous phase [73,75,76].

A single-step extraction of blood samples with ethyl acetate–isopropyl alcohol (9:1) is reportedly also possible [64]. The organic phase was evaporated and the residue redissolved in methanol and directly injected. Unfortunately, no examples were given of the 'cleanness' of the chromatograms at high sensitivity ranges. Another approach [65,66,70,71] was to use disposable columns containing inert

material (Extrelut, Clin-Elut) to absorb the sample before exposure to an organic mixture. The eluate was then evaporated and the residue redissolved and injected. A reversed-phase ion-pair extraction with coarse C_{18} silica cartridges was reported by Moore et al. [77]. Simple methods for the analysis of brain tissue have been reported by Ishikawa et al. [69] and Raffa et al. [71].

When coulometric detectors are used, a three-electrode configuration can be applied. Detection takes place in a coulometric cell set at the optimum potential. A second electrode is placed just before the first and working at a lower potential, not suitable for measurement but useful for oxidizing interferents. A third guard electrode, run at a higher potential than the two other cells and placed before the injector, can be used to reduce the baseline noise due to oxidizable compounds in the mobile phase. This complex system has been claimed to be more selective and sensitive than amperometric detectors. To our knowledge, two papers adopting this system have recently appeared, by Svensson [79] and Derendorf and Kaltenbach [80], both using reversed-phase ion-pair chromatography. Svensson, in particular, reported the determination of morphine-6-glucuronide and normorphine, in addition to morphine.

The voltages adopted by Svensson [79] and Derendorf and Kaltenbach [80] were +300 and +450 mV at the detection cell and +220 and +250 mV at the clean-up electrode, respectively. The oxidation potential on coulometric detectors, which apparently is lower than that on amperometric detectors, using an Ag/AgCl reference electrode, according to the manufacturer depends mainly on the different reference system and arrangement responsible for a cathodic shift of about 280 mV. The limits of detection achieved by Svensson [79] and Derendorf and Kaltenbach [80] were 0.29 and 1 ng/ml, respectively.

Despite the claims of large improvements in specificity, because of pre-oxidation of interferents sample treatment was not simplified in comparison with the methods using amperometric detection. Svensson et al. [40] used a two-step reversed-phase extraction with coarse C_{18} silica cartridges and Derendorf et al. [74] did not modify the liquid–liquid procedure adopted in previous work using an amperometric detector.

Coulometric detection appears to have advantages over amperometric detection with urine samples. White [60] first observed that too large amounts of compounds interfering with the amperometric measurement were extracted. Only Derendorf et al. [74] reported the possibility of analysing urine samples by using an amperometric detector, although the sensitivity, in comparison with plasma samples, was reduced. Both the methods using coulometric detection [79,80] have proved suitable for the analysis of urine.

Although ED seems the method of choice for the HPLC analysis of morphine, including some congeners and metabolites, problems of sample preparation still remain, especially when urine or complex matrices (hair, bile, putrefied blood) have to be investigated. To improve the specificity of the detector and its ruggedness when dealing with 'dirty' extracts, we have devised the possibility of halving the electrode potential, with only a slight decrease in sensitivity.

According to Proksa and Molnar [62], there is a very significant cathodic shift of the half-wave potential of morphine at basic pH. This phenomenon is probably

due to the ionization of the phenolic group in basic media. On this basis, we have developed a reversed-phase HPLC method with a pH-stable poly(styrene–divinylbenzene) column with a basic eluent (pH 9.5) and amperometric detection at +350 mV [82]. It has been applied to the analysis of hair extracts, proving sensitive (5 ng/ml) and reliable. Under these conditions the background current was fairly low, allowing a stable baseline even at the highest sensitivity ranges (0.2 nA f.s.), with the conditioning time reduced to 10–20 min. Only a simple one-step liquid–liquid extraction was necessary, either with chloroform–isopropyl alcohol (9:1) or with commercially available ready-to-use tubes (Toxi-Tubes A).

FINAL NOTE

We should point out that supercritical fluid chromatography is also proving capable of becoming a useful tool for morphine determination [83,84].

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