Journal of Chromatography, 507 (1990) 253–258
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1698

Direct injection high-performance liquid chromatographic assay of morphine with electrochemical detection, a polymeric column and an alkaline eluent

F. TAGLIARO* and G. CARLI
Institute of Forensic Medicine, University of Verona, Policlinico di Borgo Roma, 37134 Verona (Italy)
R. DORIZZI
Laboratory of Clinical Chemistry, Legnago Hospital, Legnago (Italy)
and
M. MARIGO
Institute of Forensic Medicine, University of Verona, Policlinico di Borgo Roma, 37134 Verona (Italy)

SUMMARY

A simple method is described for the direct determination of morphine in untreated plasma or serum, using reversed-phase high-performance liquid chromatography with amperometric electrochemical detection. A basic eluent [0.05 mol/l phosphate buffer–isopropanol–tetrahydrofuran (88:10:2) (pH 9.5)] allows both reversed-phase chromatography of morphine under ionization control conditions and its detectability at an unprotected thin-layer glassy carbon electrode at a potential of 350 mV (*vs.* an Ag/AgCl reference electrode). In addition, the alkaline mobile phase promotes the ionization of serum proteins, which, being poorly retained by the hydrophobic column packing [poly(styrene–divinylbenzene) copolymer], elute early in the chromatogram, leaving a clean baseline. Up to 50 μl of simply filtered plasma can be injected. The absolute limit of detection is 0.75 ng on-column. No interferences were observed from more than 80 opiate and non-opiate drugs. The intra- and inter-assay relative standard deviations (n = 5) were 3.2 and 6.6%, respectively, at a morphine concentration of 100 ng/ml in plasma and 0.09 and 4.2%, respectively, at the level of 500 ng/ml.

INTRODUCTION

The ability of modern liquid chromatographic systems to handle samples containing large amounts of proteins has prompted the direct high-performance liquid chromatographic (HPLC) analysis of biological fluids, such as blood plasma and serum. Although devised in the late 1970s[1], this approach has until recently been regarded as little more than a curiosity. However, given the great interest in coupling the specificity and reliability of chromatography with the operative simplicity and high sample throughput typical of the newest clinical chemistry assays, in recent times

0021-9673/90/$03.50 © 1990 Elsevier Science Publishers B.V.

much effort has been put into the development of HPLC methods suitable for plasma or serum injection.

Yoshida *et al.*[2] proposed the use of 'protein-coated ODS' and later Hagestam and Pinkerton[3] introduced the concept of the internal surface reversed phase. More recently, Shihabi and co-workers used either silica-based wide-pore[4] or polymeric columns[5] for the direct determination of carbamazepine and pentobarbital, respectively, with injections of a few microlitres of serum. A silica support with a polymeric surface in which hydrophobic pockets are shielded by a hydrophilic network (shielded hydrophobic phase) was later reported by Gisch *et al.*[6]. A different approach to the same problem was developed by Arunyanart and co-workers by using micellar mobile phases[7] and cyano or C_{18} derivatized silica columns[8]. Recently, excellent reviews on this subject were published by Westerlund[9] and Shihabi[10].

All of these different approaches proved useful particularly in therapeutic drug monitoring (TDM). Direct assays have been reported of therapeutic and potentially toxic drugs present at relatively high concentrations (>1 μg/ml) with a high molar absorptivity above 250 nm; lower wavelengths are usually precluded by interfering compounds present naturally in the serum.

Until now, there have been very few reports concerning methods for the direct HPLC assay of basic drugs, particularly morphine. It is well known that silica-based column packings are in general problematic for the reversed-phase chromatography of basic compounds, because of the residual silanols. Numerous remedies such as adjustment of the pH of the mobile phase and addition of organic amines have been proposed, often achieving limited results in terms of improvements in peak shape[11]. Moreover, in our opinion, the 'silanol effect' is a difficult point in systems dedicated to the direct injection of plasma and serum, in which the mobile phase composition (pH, ionic strength, organic modifiers) is strongly limited by the need to allow solubility in the eluent of large protein fractions throughout chromatography. In addition, as the molar absorptivity of morphine is poor above 210–220 nm and its potentially lethal levels are fairly low (ca. 0.1–1 μg/ml), 'direct injection' HPLC methods with UV detection are in practice unsuitable. The only examples of direct morphine determination in untreated biological fluids were reported by Nelson *et al.*[12] and, more recently, by Arunyanart and Cline Love[8]; however both appear ill-suited for practical use. The former, adopting a reversed-phase system with off-line analysis of the collected fractions of the eluate by immunoassay, is too complex and time consuming; the latter, using micellar chromatography with fluorescence HPLC detection, has limited sensitivity (300 ng/ml).

Recently, we developed an HPLC assay for morphine using an alkaline mobile phase (pH 9.5) and a poly(styrene–divinylbenzene) column (PLRP-S) with amperometric electrochemical detection (ED) at an oxidation potential of 350 mV[13]. Because of the high selectivity of such a low voltage, the injection of biological samples required only simple and rough extraction procedures.

Hux *et al.*[14] and Shihabi *et al.*[5] reported the use of polymeric packings (Amberlite XAD-2 and PRP-1) for achieving the direct injection of blood plasma and serum into HPLC systems. They observed that, as most plasma proteins have their isoelectric point close to neutral pH, in alkaline media they are highly charged and therefore unretained by the hydrophobic, pH-stable stationary phase. In addition polymeric matrices for basic compounds allow work in reversed-phase chromatogra-

phy under ionization control conditions, and intrinsically lack any 'silanol effect'.

On this basis, we devised the possibility of developing a 'direct injection' HPLC assay of morphine by simple adjustments of the above-mentioned HPLC–ED method, which just used a polymeric column and an alkaline mobile phase. In addition, taking advantage of the low potential (350 mV) required to oxidize morphine at basic pH, the use of highly sensitive amperometric detection seemed not to be precluded, notwithstanding the risks of electrode passivation because of the amounts of proteins injected.

EXPERIMENTAL

Apparatus

The isocratic HPLC system consisted of a Model 880 PU high-pressure pump (Jasco, Tokyo, Japan), a Model 7125 sample injector with a 50-μl loop (Rheodyne, Cotati, CA, U.S.A.), a Clar 055 column oven (Violet, Rome, Italy) and an electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon thin-layer transducer cell (LC-17A) with an Ag/AgCl reference electrode, and an LC-4B controller. An on-line 0.5-μm filter frit (Rheodyne) following the injection valve protected the column from particle contamination. The column used was a PLRP-S 300 Å, 8 μm (250 x 4.6 mm I.D.) from Polymer Labs. (Church Stretton, U.K.), packed with spherical macroporous poly(styrene–divinylbenzene) particles. A 150 x 4.6 mm I.D. column packed with 100 Å 5 μm resin from the same producer was also used in early experiments.

Reagents, standards and samples

HPLC-grade solvents, analytical-reagent grade chemicals and morphine hydrochloride from Carlo Erba (Milan, Italy) were used.

Standards of therapeutic drugs and drugs of abuse, supplied desiccated on glass microfibre discs impregnated with silicic acid (Toxi Disc Library) were purchased from Analytical Systems (Laguna Hills, CA, U.S.A.).

A stock solution of morphine (1 mg/ml) was prepared in water; working standard solutions were prepared in human plasma over the range 31–500 ng/ml.

Human blood plasma (sodium citrate) serum and 'cadaveric serum' were obtained by centrifugation for 10 min at 1500 *g*. The supernatant was stored at −20°C for assay later (within 1 month).

Control samples were preliminarily checked by a commercially available radioimmunoassay (RIA) for morphine (Diagnostic Products, Los Angeles, CA, U.S.A.) with a sensitivity of 2.5 ng/ml.

Spiked plasma samples were assayed after allowing them to stand for at least 1 h in order to allow protein binding equilibration. However, a comparison study with samples incubated for 24 h showed no differences.

Procedure

Sample preparation was limited to filtration through 0.45-μm disposable cellulose acetate filters. Frozen and/or 'dirty' samples (*e.g.*, cadaveric blood, sera containing residues of clotting or precipitates) were clarified by centrifugation, before filtration. The volume of plasma and serum injected varied from 25 to 50 μl.

Chromatographic separation was carried out with a mobile phase composed of 0.05 mol/l phosphate buffer–isopropanol (IPA)–tetrahydrofuran (THF) (88:10:2) at pH 9.5. The eluent mixture was filtered under reduced pressure through a 0.45-μm nylon 66 membrane filter (Alltech, Eke, Belgium) prior to use. The flow-rate was 1 ml/min. The column oven was set at 65°C. Under these conditions the pressure on the top of the column was in the range 80–90 kg/cm^2.

Detection was by electrochemical oxidation at a thin-layer carbon electrode using a potential of 350 mV *vs.* Ag/AgCl. Sensitivity ranges down to 1 nA full-scale were used, still with an acceptable baseline noise.

Because no extraction procedures requiring the monitoring of the recovery were involved, we used external standardization, interpolating the concentrations of unknown samples from a plot of peak height of morphine standards diluted in plasma *vs.* concentration in the range 31–500 ng/ml.

RESULTS AND DISCUSSION

Serum and plasma samples showed no evidence of precipitation when mixed with the mobile phase at pH 9.5; however a slow increase in pressure was observed after serveral injections of real samples into the HPLC system, but it was always possible to restore normal levels by replacing the on-line frit filter between injector and column. Frits were recycled by boiling in 65% nitric acid for 20 min and thoroughly rinsing with distilled water.

No evidence of protein precipitation or adsorption on the top of the column was observed, even after more than 200 injections. So far, the procedure of column regeneration by reversing the flow direction or by removing and replacing the first few millimetres of the resin, as reported by Shihabi *et al.*[5], has not been required. However, the column was always flushed overnight and during weekends with mobile phase at 0.01 ml/min, without recycling.

Under the described conditions, proteins and other endogenous compounds eluted with the 'solvent front' of the chromatogram within 9 min, allowing a clean baseline for the morphine peak. The morphine peak ($k' \approx 4$) was resolved well from matrix components and showed an acceptable asymmetry factor of 1.3 at 10% of the peak maximum, but was fairly broad. No relevant differences in the chromatographic pattern were observed when serum instead of plasma was injected. Typical chromatograms of control and spiked human plasma are shown in Fig. 1.

Sharper peaks were obtained by using acetonitrile (15%) as the organic modifier, but in this instance the solvent front was broad enough to overlap the morphine peak at the highest sensitivities. The observed poor efficiency may be dependent on both the particle size (8 μm) and pore size (300 Å) of the resin. On the other hand, Shihabi and Dyer[4,10] reported some advantages of wide-pore over narrow-pore packings when used for direct serum injection. Our experience with a 100 Å, 5 μm resin, packed in a 150 x 4.6 mm I.D. column, confirmed problems with matrix components and worsening peak shapes. Progressive clogging of the particle pores at the top of the column by the largest proteins injected, with related disturbances to band formation, could tentatively be suggested.

Possible interferences from as many as 82 opiate and non-opiate drugs at concentrations of 20 μg/ml were excluded. It should be noted that hydromorphone was

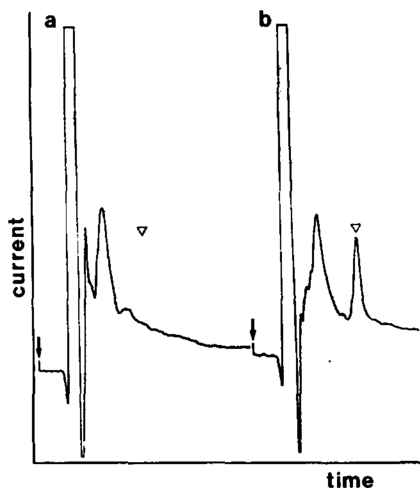

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma spiked with 250 ng/ml of morphine. Injection volume, 25 μl; sensitivity range, 2 nA full-scale deflection; chart speed, 0.5 cm/min. Other conditions as in the text. The triangles indicates morphine retention time (13.4 min) and the arrows indicate injection.

not completely resolved from morphine (α = 1.2). Complete resolution was achieved by omitting THF from the mobile phase. However, in this instance the morphine peak shape was much poorer. Also, the use of acetonitrile (15%) in place of IPA and THF in the eluent (at the same pH of 9.5) achieved a baseline separation between morphine and hydromorphone but, as noted above and elsewhere[5], this solvent did not fit well with the need for protein solubility. Nevertheless, in case of any doubt, both of these eluent mixtures can actually be used for resolving the two compounds.

Oxidation potential was chosen on the basis of a previous study on the hydrodynamic electrochemical behaviour of morphine at different pH which showed a marked cathodic shift of the voltammographic curve at increasing pH[13]. Although not fully understood, this phenomenon could be attributed to the favoured ionization of the morphine phenolic group, which would promote the oxidation of the molecule. In practice, in alkaline media electrochemical detection of morphine is possible at fairly low potentials; +350 mV was chosen as the best compromise between the morphine signal and the matrix-related noise. Under these conditions, detector selectivity and ruggedness are substantially improved, allowing relatively large amounts of proteins and other endogenous compounds, such as found in 'direct injection' methods, to be handled. No evidence of electrode passivation was observed even after 20–30 serial plasma injections per day. Electrode cleaning was intentionally omitted for 1 month without any appreciable decay of response. The background current was always in the range 2–5 nA.

The relative limit of detection (LOD) signal (= three times the noise, measured with plasma blanks) was 30 ng/ml and the absolute LOD was 0.75 ng on-column. The linearity of the assay within the range 31–500 ng/ml was described by the equation $y = -0.182 + 0.0371x$; $r = 0.99996$, where x is morphine concentration (ng/ml) and y is peak height (cm). The intra-assay relative standard deviation (R.S.D.) ($n = 5$) was 4.3, 3.2, 1.6 and 0.09% at morphine levels of 50, 100, 200 and 500 ng/ml in

plasma, respectively. The inter-assay R.S.D. (n = 5), evaluated at concentrations of 100 and 500 ng/ml, was 6.5 and 4.2%, respectively.

Eleven sera from patients admitted to a first-aid station with symptoms of heroin overdose were assayed by the presented method and the results were compared with those obtained by RIA. A good correlation was obtained (r = 0.98).

CONCLUSION

Reversed-phase HPLC at basic pH coupled with electrochemical detection at a low potential has proved able to deal with the direct injection of plasma and serum samples. Polymeric wide-pore column packings seem suitable for this purpose. On this basis, a very simple and rugged HPLC–ED method for morphine determination was developed, which seems particularly useful in clinical toxicology. Although less sensitive than other methods requiring liquid–liquid or solid–liquid extractions[15], the present method is by far the most sensitive of those without any extraction step.

This work strongly supports the ability of amperometric electrochemical detection, in flow systems, to deal with untreated body fluids, which too often are considered incompatible with electrodes. This approach could provide 'direct injection' HPLC methods with the sensitivity the lack of which is claimed to be their main disadvantage. The use of unprotected electrodes seems possible only for compounds amenable to electrolysis at relatively low potentials, such as morphine. Otherwise, electrode protection with size-exclusion membranes could be adopted, although this would cause a significant loss of signal. Of course, these considerations apply particularly to thin-layer electrodes; 'coulometric' detectors seem intrinsically less suitable for these purposes, as endogenous proteins are more likely to foul porous electrodes by irreversible adsorption.

REFERENCES

1 D. J. Popovich, E. T. Butts and C. J. Lancaster, *J. Liq. Chromatogr.*, 1 (1978) 469–478.
2 H. Yoshida, I. Morita, G. Tamai, T. Masujima, T. Tsuru, N. Takai and H. Imai, *Chromatographia*, 19 (1984) 466–472.
3 H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757–1763.
4 Z. K. Shihabi and R. D. Dyer, *J. Liq. Chromatogr.*, 10 (1987) 2383–2391.
5 Z. K. Shihabi, R. D. Dyer and J. Scaro, *J. Liq. Chromatogr.*, 10 (1987) 663–672.
6 D. J. Gisch, B. Feibush and R. Eksteen, poster at the *17th International Symposium on Chromatography, Vienna, September 25–30, 1988*, Abstracts, I P 30.
7 F. J. DeLuccia, M. Arunyanart and L. J. Cline Love, *Anal. Chem.*, 57 (1985) 1564–1568.
8 M. Arunyanart and L. J. Cline Love, *J. Chromatogr.*, 342 (1985) 293–301.
9 D. Westerlund, *Chromatographia*, 24 (1987) 155–164.
10 Z. K. Shihabi, *J. Liq. Chromatogr.*, 11 (1988) 1579–1593.
11 D. Chan Leach, M. A. Stadalius, J. S. Berus and L. R. Snyder, *LC·GC Int. Mag. Liq. Gas Chromatogr.*, 1 (1988) 22–30.
12 P. E. Nelson, S. M. Fletcher and A. C. Moffat, *J. Forensic Sci. Soc.*, 20 (1980) 195–202.
13 F. Tagliaro, G. Carli, F. Cristofori, G. Campagnari and M. Marigo, *Chromatographia*, 26 (1988) 163–167.
14 R. A. Hux, H. Y. Mohammed and F. F. Cantwell, *Anal. Chem.*, 54 (1982) 113–117.
15 F. Tagliaro, D. Franchi, R. Dorizzi and M. Marigo, *J. Chromatogr.*, 488 (1989) 215–228.