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Automated sample preparation by on-line dialysis and trace enrichment

Analysis of morphine, 6-monoacetylmorphine, codeine, ethylmorphine and pholcodine in plasma and whole blood by capillary gas chromatography and capillary gas chromatography–mass spectrometry

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ABSTRACT

A fully automated sample preparation method for the determination of five opiates in human plasma and whole blood is described. The technique combines dialysis and trace enrichment prior to gas chromatography and gas chromatography–mass spectrometry. Dialysis and trace enrichment on a polymer column was shown to be a highly reliable method for sample preparation. The method can be used, after minor modification, to determine other basic drugs in plasma and whole blood. The method demonstrates the potential of the automated sequential trace enrichment of dialysate (ASTED) system for automated sample preparation.

INTRODUCTION

The determination of opiates in small samples of blood is a problem commonly encountered in forensic toxicology. Unspecific immunological methods are commonly used for screening of the samples, but sensitive, specific and simple confirmation methods are required to separate the different opiate drugs and metabolites, to distinguish between illegal and legal use of drugs. The pretreatment of biological samples is an important step in the analysis and very often a time-consuming process. Current methods for sample preparation often include several manual steps, *e.g.* precipitation, centrifugation, extraction and concentration. By using on-line dialysis as a purification step prior to the chromatographic analysis, whole blood and plasma analysis is considerably simplified and fully automated sample preparation methods can be developed [1].

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Automated sample preparation minimises manual handling of biological samples which are potentially contagious. Only low-molecular-mass compounds diffuse through the dialysis membrane and the analytes collected in the dialysate are concentrated on a trace enrichment column. This is the principle of the fully automated AST-ED (automated sequential trace enrichment of dialysate) system. The purpose of this study was to develop a fully automated sample preparation method for the simultaneous analysis of several compounds in plasma and whole blood based on the ASTED system (Fig. 1). The ASTED has so far been connected on-line to an high-performance liquid chromatography (HPLC) system. We recently reported a fully automated method for the analysis of alkaline drugs based on the AST-ED system and HPLC [2]. In forensic toxicology, capillary gas chromatography (GC) and capillary GC–mass spectrometry (MS) are often the preferred methods for analyte confirmation and quantification. The purpose of this study was also to demonstrate the potential of the ASTED system for automated sample preparation in general combined with other chromatographic techniques besides HPLC. The ASTED system was connected on-line to a fraction collector for further analysis by capillary GC and capillary GC–MS. Five opiates, morphine, codeine, ethylmorphine, pholcodine and 6-monoacetylmorphine, were chosen as the compounds to be analysed by the method developed.

EXPERIMENTAL

Chemicals

Pholcodine (Ph) was obtained from Weiders Farmasøytiske (Oslo, Norway). Morphine hydrochloride (M), codeine phosphate (C), ethylmorphine hydrochloride (E) and nalorphine hydrochloride (N) were purchased from Norsk Medisinaldepot (Oslo, Norway). 6-Monoacetylmorphine hydrochloride (6-MAM) was synthesised at the National Institute of Forensic Toxicology (Oslo, Norway). Ammonia, Triton X-100 and sodium acetate, all of p.a. grade, were from Merck (Darmstadt, Germany). Sodium azide of AnalaR grade was purchased from BDH (Poole, UK). Ampules (1 ml) of N,O-bis(trimethylsilyl)trifluoroacetamid (BSTFA) and pentafluoropropionic anhydride (PFPA) were supplied by Supelco (Bellefonte, PA, USA). Acetonitrile was of HPLC grade from Fisons (Loughborough, UK). *n*-Butylacetate was of glass-destilled grade from

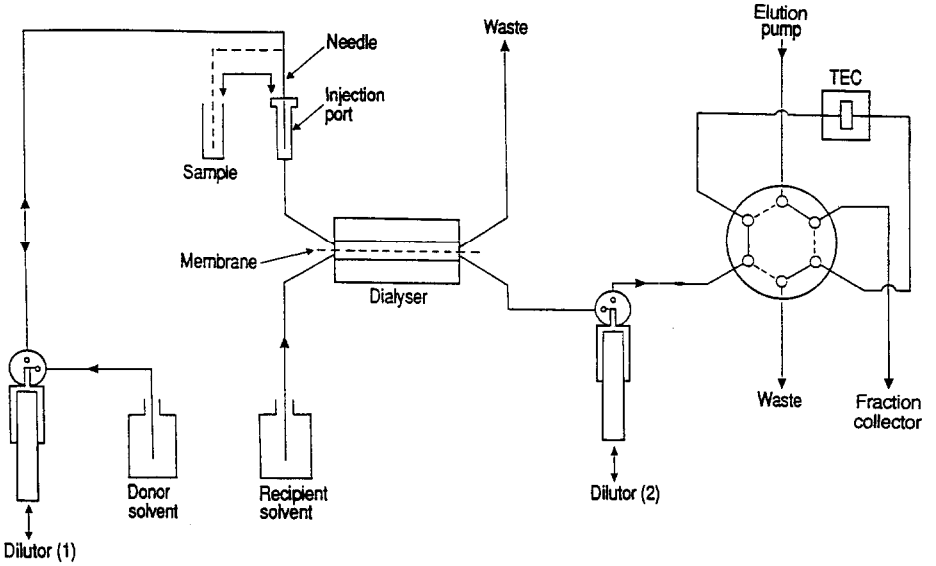

Fig. 1. Schematic diagram of the automated ASTED system.

Ratherburn (Walkerburn, UK). HPLC-grade water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Preparation of standards

Stock standard solutions (2 μmol/ml) of Ph, M, C, E, 6-MAM and N (internal standard (I.S.)) were prepared in water. Working standard solutions of all six compounds (0.2–5.0 nmol/ml) were prepared from the stock standard solutions. Plasma was separated by centrifuging citrated whole blood for 15 min at 1920 *g*. Spiked plasma and whole blood samples (0.2–5.0 nmol/ml) were prepared from the stock standard solutions and kept at −24°C until analysis.

ASTED method

The sample preparation system shown in Fig. 1 was a Gilson ASTED unit (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of a Model 231 autosampling injector, two Model 401 diluters equipped with 1-ml syringes and a flat-bed dialyser with a donor channel volume of 370 μl and a recipient channel volume of 650 μl, fitted with a cuprophane membrane of 15 000 molecular mass cut-off. A Model 7010 automated six-port valve (Rheodyne, Berkeley, CA, USA) connected a trace enrichment column either with the recipient channel of the dialyser or with the solvent used to elut the compounds retained by the trace enrichment column. A Shimadzu (Kyoto, Japan) Model LC-9A solvent delivery pump was used to elut the trace enrichment column after dialysis. This pump was programmable and able to communicate with the ASTED system. The eluates were collected by a Gilson Model 201 fraction collector connected to the ASTED. The trace enrichment column (10 mm × 2 mm I.D.) from Chrompack (Middelburg, The Netherlands) was packed with 36-μm polystyrene–divinylbenzene from Dyno Particles (Lillestrøm, Norway).

ASTED procedure

Nalorphine hydrochloride was used as I.S. and was manually added to all plasma and whole blood samples prior to dialysis to a final concentration of 1 nmol/ml.

Sample loading. One diluter was used to inject plasma or whole blood into the donor channel of the dialyser cell, with the six-port valve in the load position. The sample was held static in the donor channel.

Dialysis and trace enrichment. The sample was held static in the donor channel while the other diluter pulsed 5.0 ml of 0.1 m*M* ammonia (pH 9.7) through the recipient channel of the dialyser and into the trace enrichment column, with the injection valve still in the load position. The recipient solution was transported through the dialyser in pulses of 650 μl, corresponding to an average flow-rate of 0.56 ml/min. The total dialysis time was 9 min.

Elution and purging. On switching the six-port valve to the inject position, the analytes were back-flushed from the trace enrichment column by acetonitrile. The acetonitrile was pumped through the trace enrichment column at a flow-rate of 1 ml/min. The first 100 μl of the eluate was discarded and the next 700 μl was automatically collected in glass vials by the fraction collector. The recipient side of the dialyser was washed with 2 ml of 0.1 m*M* ammonia (pH 9.7) (flow-rate 6 ml/min). The donor side of the dialyser was simultaneously washed with 4 ml of 0.02 *M* sodium acetate solution containing 100 mg/l Triton X-100 and 50 mg/l sodium azide (flow-rate 6 ml/min).

Regeneration. After 1.5 min, the six-port valve was switched back to the load position to bring the trace enrichment column back to the recipient channel of the dialyser. After regeneration of the trace enrichment column with 1 ml of 0.1 m*M* ammonia (pH 9.7) (flow-rate 0.56 ml/min), the next sample was injected into the dialyser. Automated injections were performed every 15 min. The eluates were evaporated under nitrogen in an electrical heating block at 40°C. The dry residues were immediately derivatized.

Derivatization GC

To the dry residues were added 90 μl of BSTFA–acetonitrile (1:2, v/v). The capped vials were heated at 60°C for 20 min. The reaction mixtures were evaporated to dryness under nitrogen

(60°C) and dissolved in 50 μl of *n*-butyl acetate; 1 μl was injected into the gas chromatograph.

Derivatization GC–MS

To the dry residues were added 80 μl of PFPA instead of BSTFA, but otherwise the samples were treated in the same manner.

Gas chromatography

An HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) was equipped with a split/splitless capillary HP 7673A autoinjector and a nitrogen–phosphorus detector (NPD) operated in the nitrogen mode. The signals were received on an HP 3396 Series II integrator. The column was an HP Ultra1 cross-linked methylsilicone capillary column (12 m × 0.2 mm I.D., film thickness 0.33 μm). The samples were injected splitless and the splitter was reopened after 30 s. The detector temperature was set at 300°C and the injection port temperature at 250°C. The initial oven temperature of 120°C was held for 30 s, then increased at 40°C/min to 220°C, at 4°C/min to 244°C and at 40°C/min to 300°C, the final temperature being held for 3.5 min. Helium was used as carrier gas at a flow-rate of 2 ml/min. The detector gases were hydrogen (3.5 ml/min) and air (98 ml/min). Helium was used as make-up gas at a flow-rate of 30 ml/min [3].

Gas chromatography–mass spectrometry

An HP 5971 Series mass selective detector was connected to an HP 5890 Series II gas chromatograph. The capillary column was the same as used for the GC analysis. The mass spectrometer was operated in the electron-impact mode at 70 eV with an ion source temperature of 200°C. Selected ions were monitored at *m/z* 282 and 445 for codeine–PFP, *m/z* 296 and 459 for ethylmorphine–PFP, *m/z* 414 and 577 for morphine–PFP and *m/z* 414 and 473 for 6-MAM–PFP [4,5]. The samples prepared by on-line dialysis were compared to spiked whole blood standards prepared by solid-phase extraction, the method currently used at the National Institute of Forensic Toxicology [4,5]. Pholcodine was not analysed by GC–MS since this is confirmed by GC at the Institute.

Validation of the procedure

The calibration graphs, accuracy and precision of the method were evaluated by analysis of manually spiked plasma and whole blood samples. The calibration graphs were based on internal standard calibration, measuring peak-height ratios *versus* concentration in plasma or whole blood.

RESULTS AND DISCUSSION

Trace enrichment

During method development both silica based C_{18} and polystyrene–divinylbenzene materials were investigated for trace enrichment. In this study 0.1 m*M* ammonia pH 9.7 was selected as the recipient solution to ensure retention on a reversed-phase trace enrichment column (TEC). The silica based C_{18} material gave satisfactory clean-up and recovery, but the high pH (9.7) in the recipient solution gave an unacceptable short column life-time. The polymeric packing material was chosen due to its complete recovery of the analytes and satisfactory clean-up as well as its stability at the high pH. No breakthrough of any of the compounds was observed when up to 10 ml of 0.1 m*M* ammonia was pumped through the TEC. The TEC was used for several hundred analyses without any sign of degradation.

Evaporation of the elution solvent was a requirement before derivatisation and GC analysis. Several organic solvents and mixtures were investigated as elution solvents. Acetonitrile was chosen as the elution solvent because it gave complete recovery of all the analytes and the internal standard and was easily evaporated. No hydrolysis of 6-MAM was detected during the short time of exposure to alkaline pH.

Dialysis

We recently reported pholcodine to adsorb to the cellulose acetate membrane [2] and that flushing the donor channel with 0.02 *M* acetate buffer (pH 6) between analyses ensured the absence of a

carry-over effect. Because of the adsorption of pholcodine, the donor channel was washed with 4 ml of 0.02 *M* acetate buffer (pH 6.0) containing surfactants between analyses. None of the other compounds were found to adsorb to the dialysis membrane. The detergent Triton X-100 was added to reduce surface tension and ensure reproducible results. This washing step resulted in a robust sample pretreatment without any carry-over between analyses as shown in Figs. 2 and 3 where the blank sample was analysed directly after the spiked sample.

Dialysis efficiency

In a fixed dialysis cell the efficiency is influenced by the dialysis time and the concentration gradient across the membrane. By maintaining a steep gradient across the membrane such as in static-pulsed dialysis or static-continuous dialysis, high dialysis efficiencies are obtained in a short time [6,7]. In this study the sample was held static in the donor channel of the dialyser while 5.0 ml of 0.1 m*M* ammonia (pH 9.7) were delivered in pulses of 650 μl through the recipient channel. In this procedure ammonia diffused through the membrane and into the sample which resulted in reduction of the protein–opiate binding without precipiation of the proteins. The recovery of the compounds from both plasma and whole

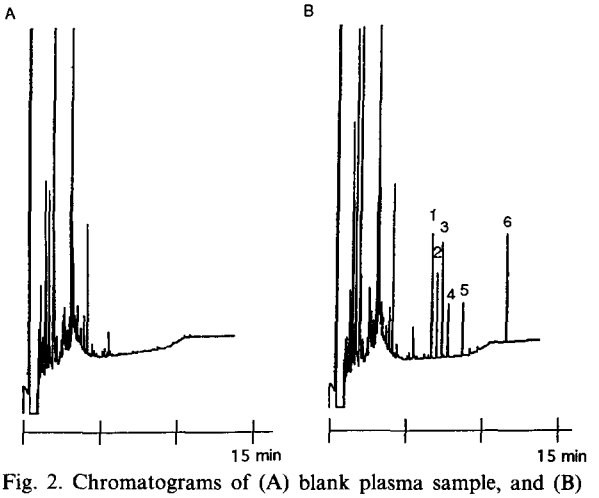

Fig. 2. Chromatograms of (A) blank plasma sample, and (B) plasma spiked with 1 nmol/ml of: 1 = codeine, 2 = ethylmorphine, 3 = morphine, 4 = 6-MAM, 5 = nalorphine (I.S.), and 6 = pholcodine.

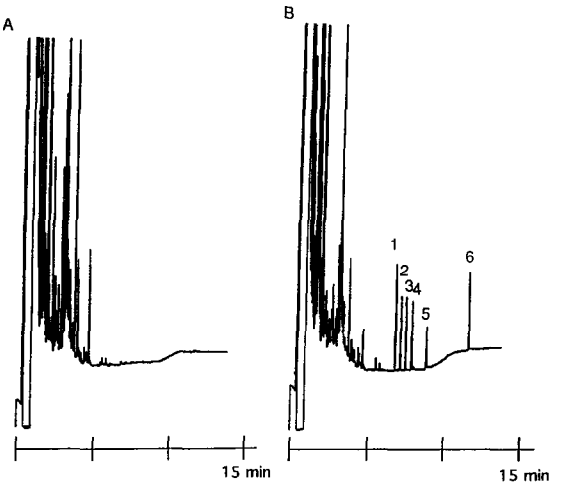

Fig. 3. Chromatograms of (A) blank whole blood, and (B) whole blood spiked with 1 nmol/min of: 1 = codeine, 2 = ethylmorphine, 3 = morphine, 4 = 6-MAM, 5 = nalorphine (I.S.) and 6 = pholcodine.

blood was between 66 and 80% when the sample was dialysed for 9 min. When the samples were dialysed with water as recipient the dialysis recovery was in the range of 30–40% which clearly demonstrates the effect of ammonia upon dialysis efficiency.

GC separation

A satisfactory separation of the compounds and endogenous substances in plasma and whole blood was achieved on a crosslinked methylsilicon capillary column in less than 14 min. Figs. 2 and 3 show chromatograms of drug-free plasma, plasma spiked with 1 nmol/ml of all compounds, drug-free whole blood and whole blood spiked with 1 nmol/ml of all compounds. The method resulted in very clean chromatograms with no interferences eluting in the vicinity of the opiates.

MS results

Selected-ion monitoring (SIM) chromatograms of morphine and codeine in whole blood (0.1 nmol/ml) are shown in Fig. 4. Selected ions were monitored at the same retention times as spiked reference standards prepared by solid-phase extraction. By using deuterium labelled drug homologues as internal standards (I.S.) opiate quantification and confirmation can be performed [4].

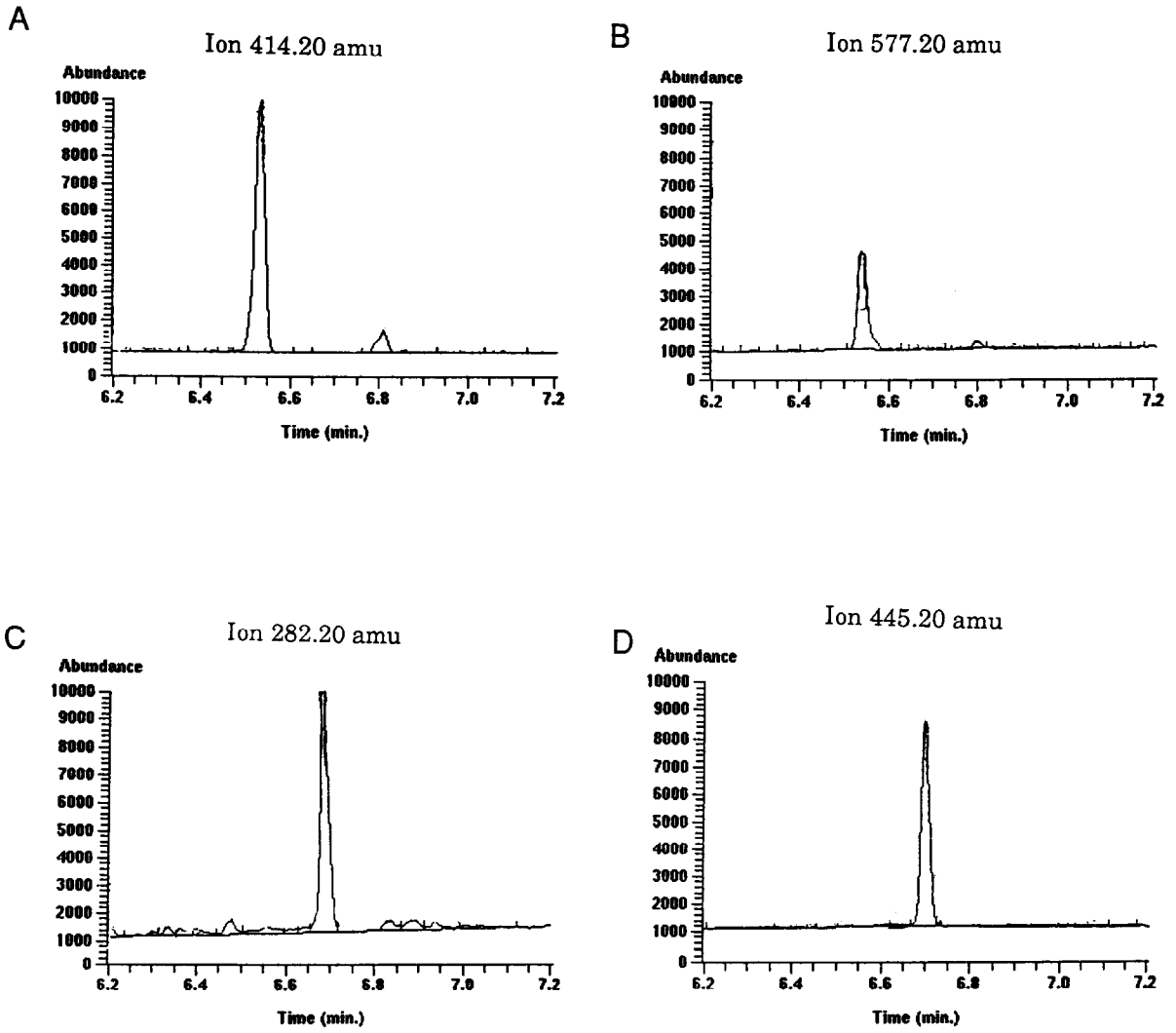

Fig. 4. Selected-ion monitoring chromatograms of morphine (A and B) and codeine (C and D) in a real whole blood sample with a concentration of 0.1 nmol/mol.

Validation of the method

The calibration graphs for plasma and whole blood were based on peak-height measurements *versus* the peak-height of the I.S. for all compounds. The calibration graphs were linear in the concentration range 0.2–5.0 nmol/ml with correlation coefficients $r = 0.9987–0.9998$ for plasma and whole blood. Tables I and II show the intra- and inter-assay variations of the method. The inter-assay variations were calculated from analyses performed on five non-consecutive days. The relative standard deviations were satisfactory and the method was shown to be highly reproducible.

Limit of detection

The limit of detection is determined by the sample size injected into the dialyser and the dialysis time. In this method a dialyser with a 370-μl donor channel volume was selected. The limit of detection for GC analysis at a signal-to-noise ratio of 3 (S/N = 3) was 0.1 nmol/ml for 6-MAM, 0.05 nmol/ml for morphine, codeine, ethylmorphine and pholcodine, both in plasma and whole

TABLE I

INTRA-ASSAY VARIATIONS AFTER ASTED SAMPLE PREPARATION, EXPRESSED AS MEAN OF PARALLEL SAMPLES ± STANDARD DEVIATION (S.D.) AND RELATIVE STANDARD DEVIATION (R.S.D.)

Opiate (n = 5)	Calculated concentration (nmol/ml)	Plasma		Whole blood	
		Measured concentration (mean ± S.D.) (nmol/ml)	R.S.D. (%)	Measured concentration (mean ± S.D.) (nmol/ml)	R.S.D. (%)
Codeine	0.2	0.194 ± 0.008	4.3	0.202 ± 0.008	4.1
	1.0	1.130 ± 0.020	1.8	0.981 ± 0.014	1.4
Ethylmorphine	0.2	0.175 ± 0.005	2.7	0.181 ± 0.008	4.3
	1.0	0.967 ± 0.015	1.5	0.972 ± 0.021	2.2
Morphine	0.2	0.203 ± 0.006	2.8	0.185 ± 0.007	3.7
	1.0	0.976 ± 0.030	3.0	1.017 ± 0.026	2.6
6-MAM	0.2	0.196 ± 0.004	1.9	0.192 ± 0.008	4.1
	1.0	1.110 ± 0.014	1.3	1.126 ± 0.045	4.0
Pholcodine	0.2	0.206 ± 0.008	3.8	0.203 ± 0.004	2.1
	1.0	0.984 ± 0.042	4.3	0.947 ± 0.034	3.6

blood. The limit of detection was considered satisfactory for the determination of all compounds. If necessary, the limit of detection can be improved by using a dialyser with a larger donor channel volume, *e.g.* by coupling two dialysers in series.

CONCLUSIONS

A fully automated sample preparation method for the determination of five opiates in human plasma and whole blood has been developed. The method includes on-line dialysis and column-

TABLE II

INTER-ASSAY VARIATIONS AFTER ASTED SAMPLE PREPARATION, EXPRESSED AS MEAN OF PARALLEL SAMPLES ± STANDARD DEVIATION (S.D.) AND RELATIVE STANDARD DEVIATION (R.S.D.)

Opiate (n = 5)	Calculated concentration (nmol/ml)	Plasma		Whole blood	
		Measured concentration (mean ± S.D.) (nmol/ml)	R.S.D. (%)	Measured concentration (mean ± S.D.) (nmol/ml)	R.S.D. (%)
Codeine	0.2	0.179 ± 0.013	7.4	0.192 ± 0.010	5.3
	1.0	1.200 ± 0.035	2.9	0.948 ± 0.034	3.6
Ethylmorphine	0.2	0.250 ± 0.017	6.8	0.204 ± 0.013	6.6
	1.0	1.101 ± 0.053	4.8	0.958 ± 0.034	3.6
Morphine	0.2	0.176 ± 0.014	7.7	0.186 ± 0.013	7.2
	1.0	1.140 ± 0.063	5.5	1.008 ± 0.002	2.1
6-MAM	0.2	0.188 ± 0.073	3.9	0.189 ± 0.009	4.7
	1.0	1.009 ± 0.030	3.0	1.011 ± 0.031	3.1
Pholcodine	0.2	0.202 ± 0.012	6.1	0.182 ± 0.010	5.6
	1.0	0.993 ± 0.045	4.5	1.065 ± 0.040	3.8

switching and was shown to be highly reliable. The method has successfully been used for automated sample preparation prior to GC and GC–MS analysis. The method can also be used, with minor modification, for the determination of other basic drugs in human plasma and/or whole blood. About 100 plasma and/or whole blood samples can be analysed in 24 h.

REFERENCES

1 N. C. van de Merbel, J. J. Hagman and U. A. Th. Brinkman, *J. Chromatogr.*, 634 (1993) 1.
2 A. T. Andresen, M. Krogh and K. E. Rasmussen, *J. Chromatogr.*, 582 (1992) 123.
3 M. Johansen, K. E. Rasmussen and A. S. Christophersen, *J. Chromatogr.*, 532 (1990) 277.
4 H. Gjerde, U. Fongen, H. Gundersen and A. S. Christophersen, *Forensic Sci. Int.*, 51 (1991) 105.
5 J. Schuberth and J. Schuberth, *J. Chromatogr.*, 490 (1989) 444.
6 A. T. Andresen, P. B. Jacobsen and K. E. Rasmussen, *J. Chromatogr.*, 575 (1992) 93.
7 M. M. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, *J. Chromatogr.*, 500 (1990) 453.