Determination of Morphine and Codeine in Blood and Bile by Gas Chromatography with a Derivatization Procedure

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Abstract

Two gas chromatographic methods for the simultaneous quantitation of morphine and codeine in blood and bile from cases of opiate-related deaths are described. Both methods employ simple solvent extraction followed by hexane–ethanol partitioning clean-up and use nalorphine as the internal standard. The first method relies on the formation of trimethylsilyl derivatives and detection with a nitrogen–phosphorous detector. The second method involves the formation of heptafluorobutyryl derivatives and detection with an electron capture detector. Both methods are sensitive, able to detect down to 0.04 μg/mL of morphine and 0.1 μg/mL of codeine. Their wide linear dynamic ranges cover from low therapeutic to lethal levels for both morphine and codeine. The methods are amenable to batchwise operation and each analysis can be completed within three hours. The results of both methods correlate very well. The trimethylsilyl derivatives can be hydrolyzed and rederivatized to form heptafluorobutyryl derivatives for the second method, which then serves to confirm the results of the first method. Pholcodine, another common opiate, can likewise be determined. Average recovery was 80% for blood morphine and codeine and 60% for bile morphine and codeine.

Introduction

There is extensive literature on morphine determination in biological samples, including immunoassay (1–3), liquid chromatography (LC) (4–6), and gas chromatography (GC) (7–19). Most immunoassay techniques detect both the free and conjugated morphine, and the results do not necessarily correlate with chromatographic techniques in which samples may not have undergone prior hydrolysis. Furthermore, the specificity of immunoassay techniques is often debatable. LC has been widely adopted for morphine analysis, but it generally requires either postcolumn derivatization or specialized detectors, such as an electrochemical detector, to attain the sensitivity required for therapeutic monitoring. In addition, LC columns are comparatively less reproducible than GC columns. LC columns of the same brand from the same manufacturer may have retention characteristics sufficiently different to warrant significant modification of the separation conditions.

A brief summary of the GC methods commonly used for morphine determination in biological specimens is given in Table I. Most of the methods require derivatization with silylating or acylating agents to improve the morphine elution profiles. Silylated morphine has been detected with a flame ionization detector (FID). However, the sensitivity is barely sufficient for therapeutic monitoring unless a large sample size (e.g. more than 5 mL of blood) was used. Electron capture detection (ECD) offers a much higher sensitivity towards the halogen-containing morphine derivatives. However, there have been no reports of applying ECD for the simultaneous determination of morphine and codeine with nalorphine as an internal standard, and using heptafluorobutyric anhydride as the derivatizing agent. Mass spectrometric detection offers very high sensitivity and specificity for morphine and codeine. However, the expensive instrumentation involved may prohibit its use in some laboratories. Nitrogen–phosphorus detection (NPD), also known as flame thermionic detection (FTD), is rarely used for morphine determination, though it should provide a higher selectivity and sensitivity towards morphine than FID with only a small increase in expense.

This paper reports the use of ECD and NPD for the simultaneous determination of morphine and codeine in blood and bile with nalorphine as the internal standard. It also describes how the two detection methods can be coupled together to increase the reliability of the determination.

Materials and Methods

Instrumentation

A Shimadzu gas chromatograph Model GC-9AFTP housing a Hewlett-Packard HP-1 fused-silica macrobore column (10 m × 0.53 mm i.d.) was employed for the NPD detection of trimethylsilyl (TMS) derivatives. The NPD bead current and flow rates of the detector gases (air, H_2) were set according to the manufacturer's specifications. Helium was used as carrier gas at a flow rate of 15 mL/min. No detector make-up gas was required.

A Shimadzu gas chromatograph Model GC-16A equipped with an ECD and housing a glass column (2 m × 2 mm i.d.)

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packed with premixed stationary phase (1.5% OV-17 + 1.95% OV-202 on Chromosorb W-HP 100/120 mesh) obtained from Alltech Associates/Applied Science Laboratory was used for the ECD detection of heptafluorobutyryl (HFB) derivatives. The ECD current was set to 0.5 nA and nitrogen was used as the carrier gas at a flow rate of 50 mL/min.

For either GC units, a Shimadzu CR-3A integrator was used for signal handling. A Denley Spiramix 5 (Sussex, England) roller-mixer for gentle liquid–liquid extractions, a Centaur Model 2 centrifuge (MSE) to assist phase separation, and a Thermolyne Maxi-Mix vortex mixer were used during extraction. 10-mL and 30-mL capacity polypropylene tubes from Sarstedt Co. were used as extraction vessels for blood and bile, respectively. A Techne Dri-Block® 08-3 SC-3 sample concentrator with N_2 inlet was employed to evaporate extracts at controlled temperature under a stream of N_2. Finally, sample and reagent dispensings were performed using appropriate dispensers (Brand Transferpette®, West Germany).

Reagents

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heptafluorobutyric anhydride (HFBA) were obtained as 1-mL vials from Pierce Chemical and Alltech Associates/Applied Science Laboratory, respectively. Nalorphine hydrobromide from Sigma Chemicals was dissolved in water to obtain a working internal standard solution of about 36 µg/mL. Authentic morphine hydrochloride and codeine phosphate were obtained from British Drug House. Appropriate quantities were dissolved in water and spiked to blank blood or bile to provide calibration standards covering the ranges of 0.04–10 µg/mL for blood and 0.4–50 µg/mL for bile.

Other chemicals used were of reagent grade. An extracting solvent mixture was made of toluene, dichloromethane, and isobutanol in a volume ratio of 6:3:1. A biphasic mixture of hexane in equilibrium with 80% aqueous ethanol was used for partitioning clean-up of the extracts.

Sample extraction

To 2 mL of blood in a 10-mL polypropylene tube were added 50 µL of nalorphine internal standard solution, 1 mL of saturated sodium bicarbonate solution (pH 9), and 6 mL of the extracting solvent. The mixture was roller-mixed for an hour and then centrifuged at 3,400 rpm for 15 min. The upper organic layer was transferred to another polypropylene tube and evaporated to dryness under a stream of N_2 at 85°C. Then 300 µL of the 80% aqueous ethanol and 3 mL of hexane were added to the residue. The mixture was vortexed at high speed for 30 s and centrifuged at 3,400 rpm for 5 min. The upper hexane layer was aspirated to waste while the lower ethanolic layer was transferred to a GC reacti-vial and evaporated to dryness under a stream of N_2 at 85°C. The residue was ready for derivatization and GC analysis as described below.

For bile, an acid digestion step was included. One milliliter of bile was mixed with 250 µL of the internal standard, 4 mL of saturated ammonium sulfate solution, and 1.2 mL of concentrated HCl in a 15-mL glass test tube. The mixture was heated in a water bath for 30 min and filtered. The filtrate was collected in a 30-mL polypropylene tube, cooled, and its pH adjusted to about 9 with 1 mL of saturated NaOH solution. Then 6 mL of extracting solvent was added, and the extraction and subsequent clean-up were as described above.

Standards for constructing the calibration curves were prepared by adding known amounts of standard morphine and codeine solutions to similar substrates known to be free of opiates. They were then processed similarly.

BSTFA derivatization and GC–NPD detection

The residue was dissolved in 100 µL of ethyl acetate, and 30 µL of BSTFA was added. The vial was tightly capped and allowed to stand at room temperature for more than 15 min. One microliter was injected into the HP-1 column. The column temperature was programmed as follows: initial temperature 220°C, initial hold 6 minutes, ramp 20°C/min to 270°C, and final hold 5 minutes. The injector and detector temperatures were both maintained at 300°C.

Table I. Summary of GC Methods for Morphine Determination in Biological Specimens*

Ref.	Year	Sample	Derivatizing agent	Detector	Internal standard	Detection limit (µg/mL)	CV (%)
7	1974	Plasma (2 mL)	TFAA	ECD	Nalorphine	0.025	Not specified
8	1974	Blood (20 mL)	BSTFA	FID	Nalorphine	Not specified	Not specified
9	1975	Blood (5 mL)	BSA	FID	Nalorphine	0.05	Not specified
10	1977	Urine (2 mL)	HFBA	ECD	Codeine	0.001	9 at 0.2 µg/mL
11	1977	Plasma (1 mL)	PFPA	ECD	3-Ethylmorphine	0.008	Not specified
12	1981	Blood (4 mL)	AA	NPD	Nalorphine	Not specified	Not specified
13	1981	Blood (1 mL)	PFPA	ECD	Nalorphine	0.001	4 at 0.06 µg/mL
14	1985	Urine (10 mL)	AA	MSD	Nalorphine	Not specified	Not specified
			TFAA	MSD	Nalorphine	Not specified	Not specified
			PFPA	MSD	Nalorphine	Not specified	Not specified
15	1986	Blood (vol not specified)	PFPA	Morphine :ECD Codeine :NPD	Nalorphine	Not specified	Not specified
					Flurazepam	Not specified	Not specified
16	1987	Blood (10 mL)	HFBA	ECD	Nalorphine	Not specified	6
17	1989	Plasma (1 mL)	TFAA	NPD	Nalorphine	0.002	3–5
18	1989	Blood (2 mL)	PFPA	MS	Deuterated analogs	0.001	Not specified
19	1989	Blood (1 mL)	PFPA	MS	Deuterated analogs	Not specified	Not specified

* HFBA = heptafluorobutyric anhydride, AA = acetic anhydride, PFPA = pentafluorobutyric anhydride, TFAA = trifluoroacetic anhydride, and BSA = *N,O*-bis(trimethylsilyl)acetamide.

If the residue was to be analyzed by the other technique such as HFBA derivatization with ECD detection as described below, 200 µL of 95% aqueous ethanol was added and the mixture evaporated to dryness at 85°C under a stream of N_2.

HFBA derivatization and GC–ECD detection

The residue was dissolved in 50 µL of ethyl acetate, and 50 µL of HFBA was added. The vial was tightly capped. The mixture was heated at 85°C for 20 min and then evaporated to dryness under a stream of N_2 at 85°C. The residue was dissolved in 200 µL of ethyl acetate, and 0.2 µL was injected into the mixed phase column operated isothermally at 200°C. The injector and detector temperatures were set at 250°C and 320°C, respectively.

Results and Discussion

Calibration curves

Typical chromatograms for the separation of the opiate derivatives under the recommended experimental conditions are given in Figures 1 and 2. Calibration curves were rectilinear over the ranges of 0–10 µg/mL for blood morphine and codeine and 0–50 µg/mL for bile morphine and codeine. All had correlation coefficients better than 0.99. The linear regressed equations were as follows:

For NPD detection:

$$\text{Blood morphine} = 0.64 \times H_m \times W / V$$

$$\text{Blood codeine} = (0.66 \times H_c - 0.32) \times W / V$$

$$\text{Bile morphine} = 0.74 \times H_m \times W / V$$

$$\text{Bile codeine} = (0.67 \times H_c - 0.25) \times W / V$$

For ECD detection:

$$\text{Blood morphine} = 0.59 \times A_m \times W / V$$

$$\text{Blood codeine} = 4.81 \times A_c \times W / V$$

$$\text{Bile morphine} = 0.68 \times A_m \times W / V$$

$$\text{Bile codeine} = (10.4 \times A_c - 5.4) \times W / V$$

where H_m and H_c are the peak height ratios of morphine and codeine relative to nalorphine; A_m and A_c are the corresponding area ratios; W is the amount of internal standard spiked and V is the volume of blood or bile used.

Peak height ratios were used for NPD detection, because these were found to give better correlation with the drug concentrations than area ratios. The analyte peaks were integrated as tailing peaks rather then vertically truncated peaks.

Unlike morphine and nalorphine, codeine has only one hydroxy function for derivatization with HFBA. Its response towards ECD was therefore much poorer compared with that for morphine.

It was determined from the calibration standards that the minimum detectable quantities of blood morphine and codeine were about 0.04 and 0.1 µg/mL, respectively, which are sufficiently sensitive for screening these drugs in most cases of morphine-related deaths. Although ECD should have a higher sensitivity than NPD, its sensitivity is actually compromised by the small amount of extract that should be injected into the column to avoid detector contamination.

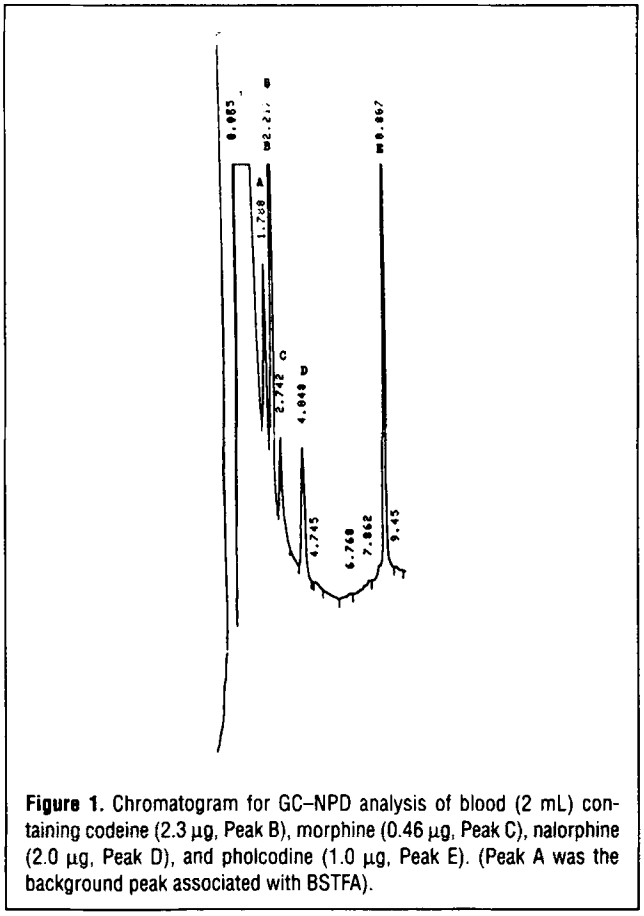

Figure 1. Chromatogram for GC–NPD analysis of blood (2 mL) containing codeine (2.3 µg, Peak B), morphine (0.46 µg, Peak C), nalorphine (2.0 µg, Peak D), and pholcodine (1.0 µg, Peak E). (Peak A was the background peak associated with BSTFA).

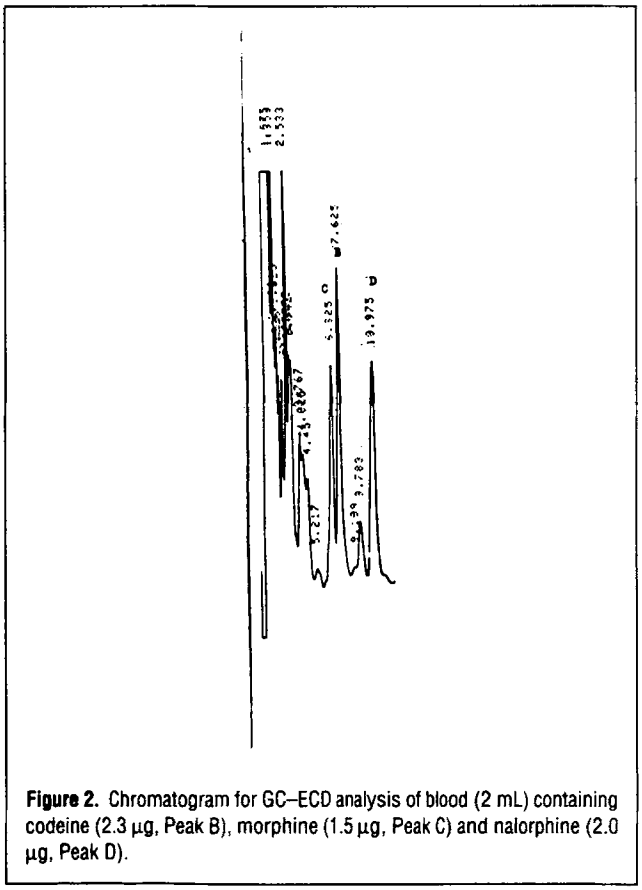

Figure 2. Chromatogram for GC–ECD analysis of blood (2 mL) containing codeine (2.3 µg, Peak B), morphine (1.5 µg, Peak C) and nalorphine (2.0 µg, Peak D).

Interference

Table II shows that other drugs commonly found in association with morphine-related deaths were well resolved from the peaks of TMS derivatives of morphine, codeine, and nalorphine, and therefore did not interfere with the NPD determination of morphine and codeine. These drugs or their HFB derivatives (if any) have much poorer sensitivity towards ECD than the corresponding derivatives of morphine and nalorphine and therefore will not interfere with morphine determination by the ECD procedure.

Simultaneous determination of pholcodine

Pholcodine is an antitussive often taken by local drug addicts. It is therefore desirable that pholcodine is screened and quantified simultaneously in addition to morphine and codeine. Table II shows that pholcodine·TMS was well resolved from other drugs. Calibration curves had been constructed for its NPD detection. The curves were linear over the ranges of 0–10 and 0–20 μg/mL for blood and bile, respectively. Detection limit was better than 0.05 μg/mL. The linear regressed equations were:

$$\text{Blood pholcodine} = (0.28 \times H_p - 0.16) \times W / V$$
$$\text{Bile pholcodine} = (0.37 \times H_p - 0.04) \times W / V$$

where H_p is the peak height ratio of pholcodine to nalorphine and W and V have the same notations as mentioned above.

Stability of the derivatives

The TMS derivatives were stable for at least a week if the containers were kept air tight. On the other hand, they could be easily hydrolyzed back to the original drugs in the presence of aqueous ethanol. After the ethanol was removed by evaporation under a stream of N_2, the opiates could be rederivatized by BSTFA with ethyl acetate as the solvent and there was no change in the peak height ratios. Alternatively, the opiates could be rederivatized with HFBA for ECD detection.

The HFB derivatives were found to be stable for about two days, after which the peaks diminished appreciably. The derivatization was apparently irreversible and rederivatization of the aged derivatized extract did not correct the problem of diminishing peak areas. There was no thermal decomposition of the derivatives observed at the low injector and column temperatures adopted.

Table II. Retention Times and Relative Retention Times of the Drugs Commonly Found in Morphine-Related Deaths

Drugs	RT (min)	RRT
Morphine·TMS	3.94	1.000
Codeine·TMS	3.11	0.788
Nalorphine·TMS	5.92	1.503
Caffeine*	0.55	0.139
Phenazone	0.60	0.152
Dextromethorphan*	1.29	0.328
Dihydrocodeine·TMS	2.58	0.655
Thebaine*	3.60	0.914
Acetylcodeine*	3.60	0.914
6-Monoacetylmorphine·TMS	4.45	1.129
Diacetylmorphine*	5.05	1.282
Quinine*	7.96	2.021
Papaverine*	8.29	2.105
Strychnine*	10.03	2.546
Pholcodine·TMS	10.75	2.728

* Did not form any trimethylsilyl derivatives and the RT and RRT refer to the underivatized drugs.

Comparison between the NPD and ECD results

Postmortem blood and bile samples from heroin addicts were concurrently analyzed for morphine and codeine by the two methods. Results are given in Table III. F test on the results shows that there is no statistically significant difference between the results of the two methods.

Some of the TMS derivatives in this study were hydrolyzed by ethanol and rederivatized with HFBA for ECD detection. The results agreed well with those obtained by direct HFBA derivatization on fresh extracts as shown in Table IV. This finding suggests that a single extract can be analyzed by BSTFA–NPD and HFBA–ECD sequentially.

Effect of the NPD bead current

The NPD sensitivity is highly dependent on the bead current apart from the H_2 and air flow rates. While the latter two parameters can be fixed, the current varies as the NPD bead ages. Table V shows that the bead current has no effect on the peak height ratios, and hence the accuracy of the determination, as long as it does not deviate too much from the operating range recommended by the manufacturer. However, it is always a good laboratory practice to adjust the current daily.

Recoveries

Using metoprolol as the external standard, recoveries of morphine and codeine in the method of BSTFA derivatization coupled to GC–NPD were monitored. Basically, the extract of blood or bile spiked with known amounts of morphine, codeine, and nalorphine was reconstituted with a known amount of metoprolol external standard solution before GC analysis. The same amounts of morphine, codeine, and nalorphine were placed in a clean vial and evaporated to dryness. The residue was similarly reconstituted with metoprolol solution to form a recovery control solution for GC analysis. The column temperature program was modified to allow a better separation of metoprolol from other analyte peaks. A typical chromatogram is shown in Figure 3. By measuring the peak height ratios of opiates to metoprolol and comparing these ratios in the extract and the recovery control, the average recoveries for blood morphine or codeine were deter-

Table III. Morphine and Codeine Levels (μg/mL) in Morphine-Related Deaths

		BSTFA derivatization with GC–NPD detection*		HFBA derivatization with GC–ECD detection*	
Case	Specimen	Morphine	Codeine	Morphine	Codeine
1	Blood	ND	ND	ND	ND
	Bile	26.7	ND	31.7	ND
2	Blood	0.06	ND	0.07	ND
	Bile	48.0	ND	43.2	ND
3	Blood	0.13	ND	0.12	ND
	Bile	11.0	ND	11.4	ND
4	Blood	0.27	ND	0.25	ND
	Bile	0.74	ND	0.70	ND
5	Blood	0.45	ND	0.39	ND
	Bile	1.2	ND	0.98	ND
6	Blood	0.79	0.30	0.74	ND
	Bile	1.1	ND	1.9	ND
7	Blood	11.6	2.1	10.2	2.1
	Bile	31.1	NA	NA	NA

* ND = Not detected; NA = Not determined

mined to be about 80%, while those for bile morphine and codeine were about 60%.

Choice of column for the GC analyses

For the analysis of the TMS derivatives, an OV-1 FSOT macrobore column afforded sufficient separation of the analytes from each other and from the blood endogenous background. Furthermore, the column under the chosen analytical conditions was able to separate most of the drugs that might be found in morphine-related deaths as shown in Table II.

Use of an OV-17 FSOT macrobore column was attempted, but the trimethylsilyl derivatives of codeine and morphine were poorly resolved under different chromatographic conditions.

OV-1 and OV-17 columns were found unsuitable for the separation of the HFB derivatives of morphine, codeine, and nalorphine. The HFB derivatives of morphine and codeine coeluted on the OV-1 column, while the OV-17 failed to separate the HFB derivatives of codeine and nalorphine. The mixed phase column recommended gave baseline separation of all three derivatives.

Choice of the extraction procedure

Both halogenated hydrocarbon–alcohol and toluene–alcohol mixtures have been widely used for the extraction of morphine from biological samples. However, emulsions are frequently produced, making phase separation difficult. The extraction procedure reported in this paper was carefully chosen, and it suffered no emulsification while offering high recoveries. Furthermore, the blood sample would not form clots, which might interfere with the extraction efficiency and reproducibility. Solvent extraction of whole blood was preferred to solid-phase extraction, which could have the problem of column blockage. The use of plastic tubes eliminated any adsorption loss that might have occured with a nonsilanized glass tube.

Table IV. Morphine Determination by Method of HFBA Derivatization with ECD Detection for Freshly Prepared Extracts and BSTFA-Derivatized Extracts following Ethanol Hydrolysis

Case	Specimen	Fresh extracts	Hydrolyzed BSTFA-derivatized extracts
2	Blood	0.07	0.07
3	Blood	0.12	0.12
	Bile	11.4	8.6
4	Blood	0.25	0.28
	Bile	0.70	0.50
5	Blood	0.39	0.44
	Bile	0.98	1.3
6	Blood	0.74	0.62
	Bile	1.9	2.0

Table V. Effects of NPD Current

Current Setting*	Peak height relative to nalorphine (2 μg)** Morphine (3.6 μg)	Codeine (4.1 μg)
65	3.19 (0.08)	4.00 (0.42)
35	3.13 (0.06)	3.99 (0.43)
19	3.04 (0.13)	3.86 (0.21)

* Unit in terms of % full scale deflection of the CR3A integrator with NPD range set at 2 and integrator attenuation at 0. The manufacturer recommends a full scale deflection of 25–50% for normal NPD operation.
** Values in brackets represent three times the coefficient of variation of five replicate determinations.

Conclusion

Morphine and codeine can be simultaneously determined by either GC–NPD using BSTFA derivatization, or by GC–ECD using HFBA derivatization. The two methods can be used sequentially to increase the reliability of the determination. The selectivity of ECD and NPD allows the use of hexane–ethanol partitioning clean-up, a less vigorous cleanup procedure as endogenous coextractants are more tolerable. Their sensitivity allows the use of a small sample size, making it useful in situations where antemortem samples are available in minuscule quantities.

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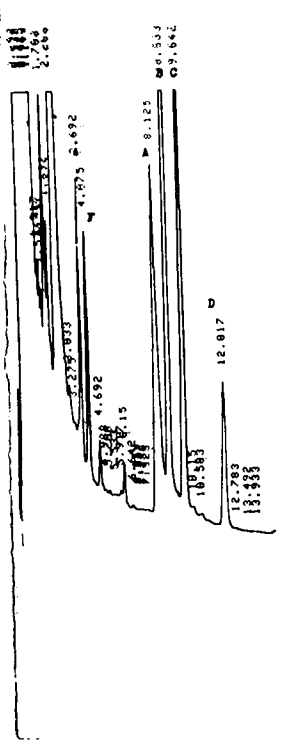

Figure 3. Chromatogram for GC–NPD analysis of blood (2 mL) containing codeine (9.1 μg, Peak B), morphine (3.8 μg, Peak C) and nalorphine (2.0 μg, Peak D) and reconstituted in 100 μL of metoprolol external standard solution (5 μg, Peak F).

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