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Simultaneous determination of in total 17 opium alkaloids and opioids in blood and urine by fast liquid chromatography–diode-array detection–fluorescence detection, after solid-phase extraction

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Abstract

A fast liquid chromatographic method with tandem diode array–fluorescence detection for the simultaneous determination of in total 17 opium alkaloids and opioids is presented. Blank blood and urine samples (1 ml) were spiked with different concentrations of a standard mixture, as well as with the internal standard, butorphanol (2000 ng/ml). After solid-phase extraction, based on weak cation exchange (Bond Elut® CBA SPE columns), the extracts were examined by HPLC–DAD–FL. By using a ''high-speed'' phenyl column (53×7.0 mm I.D., particle size 3 μm) eluted with a gradient system (A: water–methanol (90:10, v/v), B: methanol, both containing 25 m*M* triethylammoniumformate (pH_A=4.5)) all compounds could be baseline separated within 12 min. The method was validated and its applicability was demonstrated by the analysis of real-time forensic cases.

Keywords: Opium alkaloids; Opioids

1. Introduction

Opiates and their derivatives are very potent analgesics. Commonly used as therapeutic agents some of these compounds are also frequently abused as illicit drugs. Clinical and forensic testing for these compounds generally involve screening (by immunoassay) and confirmation (by chromatographic techniques), usually on biological fluids, like blood and urine.

In confirmative bio-analysis the first step is the separation and partial purification of the drugs from the biological matrix. Solid-phase extraction (SPE) has been established over recent years as a very effective method for sample pretreatment and clean-up. It offers various advantages compared to liquid–liquid extraction such as higher efficiency, selectivity and recovery, usage of smaller sample and solvent volumes, ease and convenience in handling, absence of emulsion, less time consumptive, and automation options [1]. Thus it is the method of choice for the clean-up of complex mixtures, also in opiate analysis [1–3].

To quantitate opiates and their derivatives many techniques are already available. Up to a few years ago, gas chromatography combined with mass spectrometry (GC–MS) was most often used in opiate analysis because of its sensitivity. The necessity of sample derivatisation and the cost of the technique itself were, however, restricting its applicability [4,5]. On the other hand, high-performance liquid

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chromatography (HPLC) appears as a technique that could separate a wide range of analytes without any chemical pretreatment. As such, it has become the preferred technique in most applications, using a variety of detection methods such as ultraviolet [6,7], fluorescence [8,9], electrochemical [10] or a combination of them in series [11], and most recently, mass spectrometry [12,13].

Lately, laboratories are confronted with the continuously increasing demand for higher sample throughput, thus shorter analysis time. Preferably, such methods have to be amenable to existing instrumentation, to minimize investment costs. Finally, decreased solvent consumption also became increasingly important to minimize expense and waste disposal problems [14]. A solution was given with the development of so-called ''high-speed'' columns. In comparison with the more traditional HPLC columns, the unique feature of the proposed column type is a combination of practical characteristics such as reduced column length, large internal diameter, and smaller silica particle size. On the one hand, they permit high column bed efficiency and better separation. On the other hand, higher mobile phase flow-rates are allowed without sacrificing the improved column efficiency [15].

In this paper we present a fast liquid chromatographic method with tandem diode array–fluorescence detection, after solid-phase extraction (based on weak cation exchange), for the simultaneous determination of 17 opium alkaloids and opioids in blood and urine. The method is thoroughly validated and its applicability is demonstrated by the analysis of real-time forensic cases.

2. Experimental

2.1. Materials

The opiates studied were acetyldihydrocodeine, acetylcodeine, buprenorphine, butorphanol (internal standard, I.S.), codeine, ethylmorphine, heroin (diacetylmorphine), hydrocodone, morphine, 6-monoacetylmorphine (6-MAM), methadone, naloxone, norcodeine, normethadone, normorphine, noscapine, papaverine, and thebacone, all of which were supplied by Sigma (Bornem, Belgium).

Methanol, acetonitrile, and water were all of HPLC grade (Merck-Eurolab, Leuven, Belgium). Sodium fluoride, triethylamine (purity min. 99%) and ammonium acetate (purity min. 98%) were supplied by Sigma, while formic acid (purity min. 98%) was purchased from Merck-Eurolab.

2.2. Analyte preparation

An individual standard solution of 1 g l^{-1} of each opiate was prepared in methanol or acetonitrile, according to the solubility of the solute, and stored in the dark at −20 °C until use. Under these conditions all solutions proved stable for more than 6 months. Working standard solutions, ranging from 20 ng up to 100 μg ml^{-1} (50 μl injected on column) of all opiates, were prepared by mixing an aliquot of each stock solution, evaporating the mix, at room temperature, under a constant flow of nitrogen, and redissolving the dry residue in 5 ml of LC solvent A (see Section 2.4) in an amber, volumetric flask. A 50-μg ml^{-1} solution of internal standard, butorphanol, in methanol was also prepared. We decided to use the narcotic analgesic and antitussive, butorphanol, as an internal standard since it gave an excellent separation from the opiate mixture and, as far as we know, is not available on the Belgian market. It is, however, widely used in the US in human [16] and veterinary medicine [17]. Blank blood and urine samples (1 ml) were spiked with 40 μl of a ''spike'' solution, containing 25 ng up to 50 μg ml^{-1} for each compound in methanol, resulting in a final concentration ranging from 1 ng up to 2 μg ml^{-1} of biological fluid.

2.3. Solid-phase extraction procedure

Before applying the SPE clean-up procedure on the biological fluids, both blood and urine were pretreated in a specific manner. Blood samples (1 ml) were buffered with 1 ml of 100 m*M* ammonium acetate buffer (pH=6.8–7.0) containing 2% of sodium fluoride to eliminate enzymatic degradation [18], and spiked with 40 μl of the internal standard solution. After mixing on a vortex-mixer for 20 s and 10 min of ultrason, samples were centrifuged for 5 min at 807 *g*. For urine (1 ml) the pretreatment simply consisted of spiking with the internal standard

solution (40 μl), dilution with 4 ml of water, and vortex-mixing. Subsequently, the analytes were extracted from both matrices by solid-phase extraction on 6-ml Bond Elut® CBA columns, containing 200 mg of sorbent mass (Varian, St.-Katelijne-Waver, Belgium), by means of a Lichrolut® extraction unit from Merck (Darmstadt, Germany). The columns were first conditioned with 3 ml of methanol, followed by 3 ml of water, without allowing them to dry. The samples were loaded onto individual SPE columns and washed with 3 ml of water. Columns were dried under vacuum for 20 min and analytes were eluted with 2 ml methanol, containing 25 m*M* triethylamine and 30.3 m*M* formic acid (LC solvent B, see Section 2.4). The eluates were evaporated to dryness under nitrogen gas. The residues were reconstituted in 100 μl of water–methanol (90:10, v/v), with 25 m*M* triethylamine and 30.3 m*M* formic acid (apparent pH (pH_{app})=4.5, LC solvent A, see Section 2.4). After vortex-mixing the reconstituted samples were transferred into vials containing limited volume inserts and 50 μl of each sample were injected onto the HPLC column.

2.4. HPLC–DAD–FL instrumentation and conditions

The high-performance liquid chromatography–tandem diode array–fluorescence detector system consists of a LaChrom® separation module (Merck, Darmstadt, Germany) including an L-7100 Low-Pressure Gradient Pump, L-7200 Autosampler (injection loop 100 μl), L-7360 Column Oven, and D-7000 Interface, as well as an L-7455 Diode Array Detector and L-7480 Fluorescence Detector. The system uses the D-7000 HPLC System Manager Software running under Windows NT™ version 4.0 on a Compaq Deskpro EN.

Separation was performed on a high-speed Hypersil BDS Phenyl Rocket column (53×7.0 mm I.D., particle size 3 μm) (Alltech, Lokeren, Belgium) by the following method: (A) water–methanol (90:10, v/v), (B) methanol, both containing 25 m*M* triethylamine and 30.3 m*M* formic acid (apparent pH solvent A (pH_{app})=4.5). An optimized flow of 2 ml min^{-1} was applied, the column was kept at ambient temperature and 50 μl of the reconstituted extracts were injected. The elution program started with 100% A, changed linearly to 50% A and 50% B in 10 min and was programmed further to 100% B in 2 min. The latter solvent composition was held for 1 min and the return to the initial conditions was performed within 2 min. After 2 min of equilibration the next sample was analysed. This resulted in a method in which the last eluting component, i.e. methadone, was eluted after ~12 min and a total analysis time (elution-, wash- and equilibration period) of 17 min. The diode array wavelength was set to 240 nm, changed after 8.3 min to 280 nm and returned to 240 after 0.7 min. The excitation and emission wavelength of the fluorescence detector were set to, respectively, 280 and 335 nm.

2.5. Validation

The method is fully validated by determination of the following parameters: limit of detection (LOD) and quantitation (LOQ), linearity range, within day reproducibility, accuracy, and selectivity. Regression analysis, using seven calibrator samples, ranging from 1 ng up to 2 μg ml^{-1} for each compound, is presented. Finally, the applicability of the method is demonstrated by the analysis of authentic forensic cases.

3. Results and discussion

In the first stage, a fast HPLC–DAD method was developed [19]. To reduce the analysis time and thus increase the sample throughput a high-speed column, namely a Hypersil BDS Phenyl Rocket column (53× 7.0 mm I.D., particle size 3 μm) was applied. To increase the sensitivity and selectivity for some of the opiates a fluorescence detector was connected in series to the previous configuration. Fig. 1 shows a representative separation of the compounds dissolved directly in the injection solvent (=LC solvent A, concentration 1 μg/50 μl of each compound) obtained with the dual detection system. With diode array detection, separation of all 17 opiates is complete in ~12 min. Only nine compounds demonstrated fluorescent properties under the described conditions and are separated in 11.5 min. The resolution for both detector systems is greater than 1.5 between all pairs. Combining a more universal

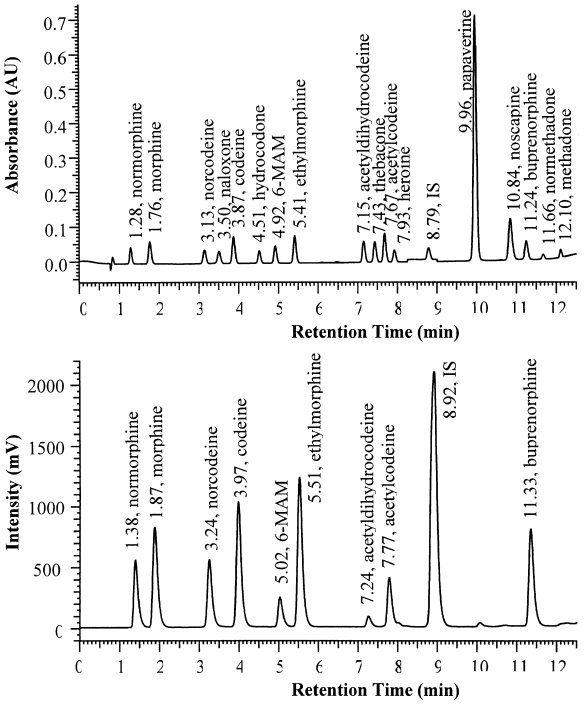

Fig. 1. HPLC–DAD (upper)–FL (lower) chromatograms of the opiate mixture (1 μg/50 μl inj.).

detector like diode array, with a more specific one such as a fluorescence detector gives the ability to get more information out of one analysis and directly confirms a positive screening result for opiates in double.

The linearity of the assay was evaluated for both detector systems. For diode-array detection, linearity was demonstrated over a concentration range of 1–5000 ng injected on column (oc) (50 μl) for all compounds. With the fluorescence detector the assay was linear over a concentration range of 1–1000 ng oc for codeine, ethylmorphine, and the internal standard, 1–2500 ng oc for norcodeine, normorphine, morphine, and buprenorphine, and finally 1–5000 ng oc for 6-MAM, acetyldihydrocodeine, and acetylcodeine. Furthermore, in none of the previous cases carry-over was seen.

Subsequently a simple but selective SPE procedure, based on weak cation exchange, for blood and urine was developed. Weak cation exchange is based on electrostatic attraction of the protonated nitrogen group in our compounds towards a negatively charged group, i.e. the bonded phase carboxylic acid ($pK_a = 4.8$). When the pH of the environment is above 4.8 the bonded phase will mainly be in negative state, attracting the positively charged opiates. However, when the pH changes to a value lower than 4.8 the carboxylic acid becomes neutral and the opiates will be eluted. A simple and easy-to-use procedure was developed which, after appropriate pretreatment of the matrices, was applied to blood and urine samples. The complete and detailed procedure is given in the Experimental (see Section 2.3). No breakthrough was seen during loading or washing while high recoveries were found after elution. Overall recoveries for both detector systems are given in Table 1. Recoveries were reproducible (C.V.<10%) and ranged for diode array detection from 66.5 to 97.0% in blood and 60.7 to 116.0% in urine. The results obtained with fluorescence detection were similar and ranged from 60.4 to 99.6% for blood and 59.6 to 102.8% for urine. Furthermore, since identical solvents to the HPLC solvents are used, the method offers possibilities as an on-line extraction.

Fig. 2 shows the DAD and FL chromatograms of spiked blood and urine samples (concentration of opiates 1 μg ml^{-1}) after the above described sample pretreatment. The chromatographic profile indicates for both matrices and both detector systems endogenous interference during the first 2 min of the analysis. This might be the explanation for the lower recoveries of normorphine and morphine in comparison with the rest of the mixture, since it is more difficult to integrate them correctly. After 2 min, no interference of endogenous compounds from blood was observed. In urine more interference was present, especially with fluorescence detection, but with a sufficiently high resolution towards the opiates so no negative effect was experienced.

The limit of detection (LOD), calculated according to the method described by the European Pharmacopoeia [20], was defined as the concentration of each component for which a signal-to-noise ratio of 3 is obtained (injected volume: 50 μl). The results, obtained for each compound of the opiate mixture are shown in Table 2. The other parameters for the quantitative validation of the method in blood with both detectors are also shown in Table 2. The results for urine are very similar and are available on file.

Table 1
Overall recovery results for spiked blood and urine (1 μg ml^{-1}) with both detector systems

	Blood DAD	Blood FL	Urine DAD	Urine FL
Normorphine	67.78	60.36	60.73	59.56
Morphine	87.15	68.65	72.83	67.07
Norcodeine	90.54	93.39	82.07	90.08
Naloxone	83.65		94.59	
Codeine	94.55	95.37	85.39	88.20
Hydrocodone	95.92		65.18	
6-MAM	96.97	94.61	96.61	100.50
Ethylmorphine	94.10	95.02	116.04	91.15
Acetyldihydrocodeine	93.60	99.59	98.96	102.82
Thebacone	86.87		93.17	
Acetylcodeine	94.58	96.05	75.80	74.59
Heroin	84.94		92.80	
Butorphanol (I.S.)	92.04	91.15	66.93	88.50
Papaverine	89.41		97.60	
Noscapine	86.79		95.89	
Buprenorphine	66.48	83.09	75.88	97.63
Normethadone	92.41		96.26	
Methadone	83.01		97.78	

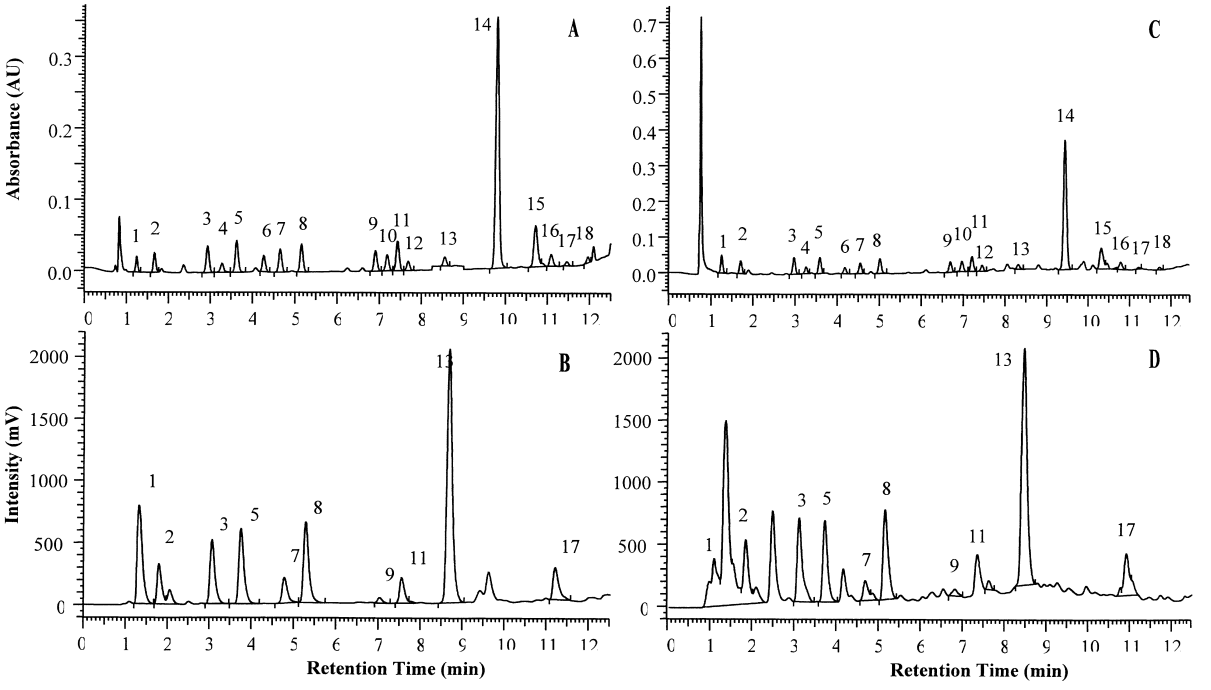

Fig. 2. HPLC–DAD–FL chromatograms obtained from the extracts of spiked blood (A, B) and urine (C, D) (concentration 1 μg ml^{-1}). (1) Normorphine, (2) morphine, (3) norcodeine, (4) naloxone, (5) codeine, (6) hydrocodone, (7) 6-monoacetylmorphine, (8) ethylmorphine, (9) acetyldihydrocodeine, (10) thebacone, (11) acetylcodeine, (12) heroin, (13) I.S., (14) papaverine, (15) noscapine, (16) buprenorphine, (17) normethadone, and (18) methadone.

Table 2
Validation results of the HPLC–DAD–FL method for blood

Compound	LOD[a] (ng ml^{-1})	Regression analysis[a,c] ($n=5$)	Within-day[a,d] (C.V.%)	Accuracy[a,e] (%)	LOD[b] (ng ml^{-1})	Regression analysis[b,c] ($n=5$)	Within-day[b,d] (C.V.%)	Accuracy[b,e] (%)
Normorphine	5.6	1.0E−03x+8.18E−02 (R^2=0.998)	5.8	94.1	2.5	0.2E−03x+0.62E−02 (R^2=0.950)	5.0	86.2
Morphine	7.6	1.6E−03x+2.29E−02 (R^2=0.998)	5.5	98.9	3.3	0.1E−03x+0.07E−02 (R^2=0.999)	5.2	88.3
Norcodeine	6.1	2.6E−03x+6.31E−02 (R^2=0.998)	5.9	97.3	2.5	0.2E−03x+0.35E−02 (R^2=0.999)	6.3	88.4
Naloxone	17.7	1.0E−03x+2.46E−02 (R^2=0.998)	5.8	96.5				
Codeine	6.3	3.1E−03x+12.9E−02 (R^2=0.998)	5.4	97.4	2.3	0.3E−03x+0.91E−02 (R^2=0.999)	5.3	88.1
Hydrocodone	14.7	1.4E−03x+6.76E−02 (R^2=0.990)	6.9	97.0				
6-MAM	9.1	2.2E−03x+6.62E−02 (R^2=0.996)	6.8	97.3	6.1	0.1E−04x+1.86E−02 (R^2=0.991)	5.9	83.9
Ethylmorphine	6.9	2.8E−03x+1.98E−02 (R^2=0.999)	6.1	98.4	1.1	0.3E−03x+0.56E−02 (R^2=0.999)	5.7	97.2
Acetyldihydrocodeine	9.8	2.0E−03x+1.28E−02 (R^2=0.998)	6.0	99.3	9.7	0.2E−04x+0.04E−02 (R^2=0.999)	5.7	83.3
Thebacone	10.7	1.9E−03x+4.31E−02 (R^2=0.998)	4.5	91.5				
Acetylcodeine	7.0	3.0E−03x+2.04E−02 (R^2=0.999)	6.1	97.5	7.0	0.9E−04x+0.06E−02 (R^2=0.999)	5.7	97.6
Heroin	15.8	1.0E−03x+0.22E−02 (R^2=1.000)	6.7	96.4				
Papaverine	1.6	27E−03x+14.0E−02 (R^2=0.999)	4.9	98.9				
Noscapine	4.1	5.4E−03x+2.61E−02 (R^2=0.999)	7.7	97.9				
Buprenorphine	8.5	1.3E−03x+7.07E−02 (R^2=0.999)	7.0	96.8	4.5	0.1E−03x+1.56E−02 (R^2=1.000)	3.5	81.9
Normethadone	52.5	0.5E−03x+0.09E−02 (R^2=0.975)	10.1	90.4				
Methadone	48.7	0.5E−03x+3.58E−02 (R^2=0.982)	10.8	82.9				

Data urine on file.
[a] DAD.
[b] FL.
[c] Mean of five calibration graphs obtained on one day.
[d] Within-day reproducibility at LOQ, coefficient of variation (C.V.%), $n=5$.
[e] Accuracy at 500 ng ml^{-1}.

With diode-array detection the following validation parameters were obtained, LOD: from 1.6 to 52.5 ng ml^{-1}; LOQ: 5.4 to 105.3 ng ml^{-1}; within-day reproducibility: C.V.% <10.8 for all compounds at LOQ. For fluorescence detection these parameters ranged from 1.1 to 9.7 ng ml^{-1} for LOD, 5.0 to 31.9 ng ml^{-1} for LOQ, and within-day reproducibility (C.V.%) was less than 6.3 for all compounds at LOQ. In addition, accuracy (at 500 ng ml^{-1}, ranging from 81.9 up to 99.3% for both detector systems), and regression analysis (using seven calibrator samples, ranging from 1 ng up to 1 μg ml^{-1}, R^2>0.990 for all except three compounds) also proved to fulfil analytical standard criteria. Furthermore, selectivity of the method was demonstrated by injection of 110 common drugs (~1 μg on column). Possible interferents were spiked in water (1 μg ml^{-1}) and injected again after solid-phase extraction. Retention data (capacity factors k') for all compounds are given in Table 3. Although co-elution of a number of compounds was seen, based on the fluorescent character and the spectral information a clear identification of the opiates was always possible. Finally, the method was successfully applied to real-time forensic cases in an effort to test the applicability of the analysis. An example of the chromatograms obtained after the analysis of a positive blood sample is given in Fig. 3.

4. Conclusion

A fast and sensitive HPLC–DAD–FL method was developed for the determination of 17 opiates in blood and urine. Solid-phase extraction, based on weak cation exchange, proved to provide excellent sample clean-up as well as reproducible and high recoveries. On the ''high-speed'' phenyl column eluted with a gradient system, all compounds could be baseline separated within 12 min. Furthermore, the dual detector system allowed to simultaneously perform a general (by diode array detection, for all opiates) and a more specific (by fluorescence detection, only nine opiates) analysis.

Table 3
Retention data for compounds evaluated as possible interferents

Compound	DAD capacity factor k'	FL capacity factor k'
Ascorbic acid	0.11	nd
Betahistidine	0.33	nd
Normorphine	**0.55**	**0.42**
Nicotine[a]	0.57	nd
Morphine	**1.09**	**0.90**
Pholcodine[a]	1.26	1.06
Acetaminophen	1.52	1.27
Theobromine[a]	2.60	2.25
Norcodeine	**2.68**	**2.28**
MDA[a]	2.94	2.63
Lidocaine[a]	3.11	nd
Theophylline[a]	3.11	2.65
Naloxone	**3.12**	**nd**
Procaine[a]	3.16	2.69
Salicyclic acid[a]	3.45	nd
Codeine	**3.55**	**3.02**
XTC[a]	3.67	3.24
Oxycodon[a]	3.76	3.28
Amoxycillin	3.83	nd
Hydrocodone	**4.34**	**nd**
EVA	4.70	nd
6-MAM	**4.83**	**4.12**
Caffeine[a]	4.96	4.24
Ethylmorphine	**5.46**	**4.66**
Metoclopramide	6.09	5.28
Tramadol	6.46	5.55
Tenoxicam	6.51	5.56
Benzoylecgonine	6.85	5.57
Fenfluramine	7.24	5.86
Phenacetine	7.26	6.19
Strychnine	7.45	nd
Phenobarbital[a]	7.50	nd
Cocaine[a]	7.60	6.42
Pethidine[a]	7.63	nd
Acetyldihydrocodeine	**7.64**	**6.53**
Cloxazolam[a]	7.64	nd
Thebacone	**7.98**	**nd**
Acetylcodeine	**8.29**	**7.53**
Tilidine[a]	8.32	nd
Chlorthalidone[a]	8.36	8.14
Doxycycline[a]	8.61	nd
Heroin	**8.62**	**nd**
Butorphanol (I.S.)	**9.71**	**8.66**
Hexobarbital[a]	9.83	nd
Citalopram	10.35	8.87
Trazodone	10.70	9.16
Zopiclone	10.74	nd
Diphenhydramine	10.76	9.24
Flecainide	10.76	9.24
Domperidone	10.84	9.38
Quinine	10.98	9.49
Secobarbital[a]	11.07	nd
Bromazepam[a]	11.16	nd
Flurazepam[a]	11.19	nd

Table 3. Continued

Compound	DAD capacity factor k'	FL capacity factor k'
Phenytoine[a]	11.24	nd
Papaverine	**11.26**	**nd**
Carbamazepine	11.49	9.84
Fentanyl	11.57	nd
Haloperidol	11.71	nd
Doxepine	11.81	10.15
Clonazepam	12.00	nd
Noscapine	**12.39**	**nd**
Chlordiazepoxide[a]	12.46	nd
Clobazam[a]	12.48	10.69
Dosulepine[a]	12.55	10.75
Flunitrazepam[a]	12.82	nd
Imipramine[a]	12.82	10.99
Methaqualone[a]	12.86	11.01
Buprenorphine	**12.87**	**11.02**
Ergotamine[a]	12.97	nd
Desipramine[a]	13.00	11.14
Fluoxetine[a]	13.03	11.16
Paroxetine[a]	13.28	11.48
Normethadone	**13.32**	**nd**
Medazepam[a]	13.33	nd
Trimipramine[a]	13.44	11.53
Amitriptyline	13.66	nd
Midazolam	13.68	nd
Brotizolam[a]	13.73	nd
Diltiazem[a]	13.79	nd
Lormetazepam	13.82	nd
Chlorpromazine	13.83	nd
Methadone	**13.95**	**nd**
Melitracen	14.11	nd
Ibuprofen	14.35	12.30
Dextromoramide	14.40	nd
Cortisol acetate	14.44	nd
Lorazepam	14.44	nd
Camazepam	14.60	nd
Bisacodyl	14.74	nd
Indomethacine	14.79	nd
Dipyridamole	14.83	nd
Disulfiram	15.00	nd
Fenthion	15.06	nd
Acetylsalicylic acid	nd	nd
Alprazolam	nd	nd
Amfepramone	nd	nd
4-Aminoantipyrine	nd	nd
Amphetamine	nd	nd
Captopril	nd	nd
Clotiazepam	nd	nd
Ephedrine	nd	nd
Ethosuximide	nd	nd
Ketamine	nd	nd
Meprobamate	nd	nd
Metamphetamine	nd	nd
Oxazepam	nd	nd
Phentermine	nd	nd

nd, not detected.
[a] Extracted.

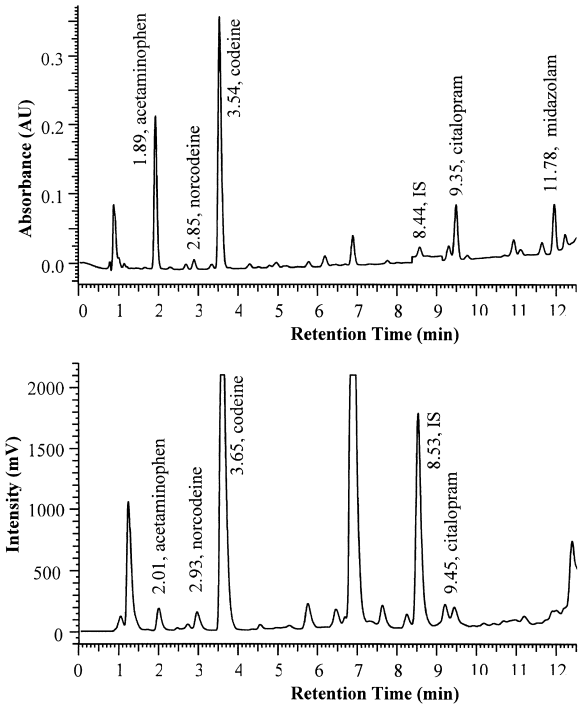

Fig. 3. Chromatogram of positive blood sample after solid-phase extraction. Besides acetaminophen, citalopram, and midazolam, it contains 436.3 ng ml^{-1} norcodeine and 8757.0 ng ml^{-1} codeine.

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