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Short Communication

Determination of codeine and its metabolites in microsomal incubates by high-performance liquid chromatography

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Abstract

A rapid and sensitive HPLC method has been developed for the determination of codeine, norcodeine and morphine in small volumes of a biological matrix, using a cyanopropyl column and a combination of coulometric and UV detection. The compounds were isolated using C_{18} solid-phase extraction cartridges prior to quantitative analysis. The limit of detection was 250 pg/ml for morphine and 5 ng/ml for both norcodeine and codeine. Recovery of each compound was greater than 90% and intra- and inter-assay precision was better than 10%. The method has been used to study the metabolism of codeine in microsomal incubations.

1. Introduction

Codeine is an opioid analgesic, used for the relief of mild to moderate pain. Codeine is metabolised by glucuronidation to codeine-6-glucuronide, N-demethylation to norcodeine and O-demethylation to morphine [1], as shown in Fig. 1. It is generally thought that the therapeutic effect of codeine is mediated primarily *via* morphine formed by oxidative metabolism, and hence morphine is the metabolite of greatest interest from a pharmacological perspective [2].

Previous approaches used for the determination of codeine and its metabolites have included GC [3,4], GC–MS [5–7], RIA [8,9] and HPLC [10–16]. HPLC has become established as the method of choice since there are no derivatization steps required, and it has no requirement for expensive/specialist equipment. Two recent HPLC methods published for the determination of codeine, norcodeine and morphine [14,15] have the disadvantage that they use time-con-

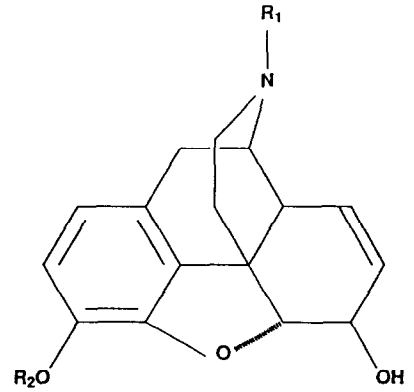

Fig. 1. Structures of codeine and its metabolites.

	R_1	R_2	M_r
Morphine	CH_3	H	285.4
Codeine	CH_3	CH_3	299.4
Norcodeine	H	CH_3	285.4

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suming liquid–liquid sample preparation procedures and require relatively large volumes of biological matrix.

The method described permits the simultaneous determination of codeine, and the codeine metabolites norcodeine and morphine in 0.1 ml of a biological matrix, using a solid-phase extraction procedure with a high and reproducible recovery of all the analytes.

2. Experimental

2.1. Chemicals

Morphine hydrochloride was purchased from May and Baker (Dagenham, UK), codeine phosphate was purchased from Boots (Nottingham, UK) and 10,11-dihydrocarbamazepine (DHCZ) was purchased from Aldrich (Poole, UK). Morphine-3-glucuronide, morphine-6-glucuronide, normorphine, norcodeine, and levorphanol were purchased from Sigma (Poole, UK). Tetrabutylammonium hydrogen sulphate (TBAHS) was purchased from Fluka (Poole, UK). Sodium dodecyl sulphate (SDS), orthophosphoric acid and all solvents were of HPLC grade and purchased from Fisons (Loughborough, UK).

2.2. Chromatography

The HPLC system consisted of a Gilson 401 dilutor, Gilson 231 automatic sample injector, Gilson 305 solvent pump, and Gilson 714 software (Gilson Medical Electronics, Villiers le Bel, France), an ABI 759A absorbance detector (Applied Biosystems Warrington, UK), set at a wavelength of 210 nm, ESA Coulochem II coulometric electrochemical detector, with a 5020 guard cell and 5011 analytical cell (ESA, Bedford, MA, USA). The coulometric detector was connected in series with the UV detector, and the potentials were set at +0.60, +0.22, and +0.45 V, for the guard cell, cell 1, and cell 2, respectively. The analytical column was prepacked with Hypersil CPS (cyanopropyl), 5 μm particle size (250 × 4.6 mm I.D.). Hypersil ODS, C_4, C_8 and SAS (short alkyl chain, C_1) columns were also evaluated (Shandon Scientific, Runcorn, UK). A guard column (20 × 2 mm I.D.) packed with pellicular ODS material (Phase Separations, Queensferry, UK) was placed between the injector and the analytical column. The mobile phase consisted of 76% (v/v) aqueous 0.05 *M* potassium hydrogen phosphate containing 1 m*M* SDS (adjusted to pH 2.5 with orthophosphoric acid), and 24% (v/v) acetonitrile. The mobile phase flow-rate was 1.0 ml/min and the volume injected was 20 μl.

2.3. Stock solutions and calibration

Morphine, norcodeine, codeine and DHCZ stock solutions were prepared in water–acetonitrile (75:25, v/v) at a concentration of 1 mg/ml, and stored at −20°C. These were diluted as required, and calibration standards containing all three compounds were prepared by adding appropriate aliquots to pH 7.4 Tris-HCl (50 m*M*) buffer. A 10-point calibration line was used with concentrations in the range 0.006–1.5 μg/ml for morphine, 0.01–2.0 μg/ml for norcodeine, and 0.1–75.0 μg/ml for codeine. The DHCZ stock solution was diluted to 25 μg/ml in water–acetonitrile (75:25, v/v), for use as the internal standard.

2.4. Extraction procedure

Extractions were performed using C_{18} Bond Elut (1 ml, 100 mg) solid-phase extraction cartridge (Varian, CA, USA), with a Vac Elut extraction apparatus which enables ten samples to be processed at a time. Both calibration standards and microsomal samples were processed in the following manner. To 0.1 ml of sample, was added 20 μl of internal standard solution (DHCZ, 25 μg/ml), 0.6 ml of carbonate buffer (pH 10.2, 0.2 *M*) and 80 μl of aqueous TBAHS solution (20 m*M*), followed by vortex-mixing after each addition. The C_{18} Bond Elut cartridge was pre-wetted with 1 ml of methanol, followed by 1 ml of de-ionised H_2O, and 1 ml of dilute carbonate buffer (pH 9.0, 5

m*M*). The sample was applied to the cartridge, and washed with 1 ml of the dilute carbonate buffer. The analytes were then eluted with 0.5 ml of 60% (v/v) aqueous 0.05 *M* potassium hydrogen phosphate containing 1 m*M* SDS (adjusted to pH 2.5 with orthophosphoric acid) and 40% (v/v) acetonitrile.

2.5. Validation

Peak-area ratios of the three compounds (morphine, norcodeine and codeine) to the internal standard (DHCZ) were calculated, and used to generate standard calibration lines of peak-area ratio *versus* drug concentration. Linear regression analysis was performed to determine the slope, intercept, and the correlation coefficient of the calibration lines.

The intra-assay precision was evaluated by spiking known amounts of the compounds into pH 7.4 Tris-HCl (50 m*M*) buffer, and six samples were determined at each of three different concentration levels for each compound, as shown in Table 1. The inter-assay precision was evaluated by analyzing duplicate samples (at the same concentration levels) on five different days.

Recovery was assessed by comparison of the absolute peak areas from the extracted samples, to the areas obtained from unextracted standard solutions prepared in mobile phase (at concentrations equivalent to 100% recovery of the compounds).

3. Results and discussion

3.1. Chromatography

The Hypersil CPS column gave the optimum chromatographic separation of codeine and its metabolites after evaluating Hypersil ODS, C_4, C_8, SAS and CPS columns. The ODS column gave long retention times (over 45 min for codeine) and poor peak shapes, when conditions were maintained to resolve all the metabolite peaks. With the SAS column the compounds were not sufficiently retained, whilst the C_4 column did not resolve the internal standard and norcodeine peaks. Improved resolution of the components was observed on the C_8 column, but

Table 1
Intra- and inter-day assay precision for codeine, norcodeine and morphine

Compound	Nominal concentration (μg/ml)	Actual concentration (μg/ml)	Precision (C.V., %)	
			Intra-day ($n = 6$)	Inter-day ($n = 10$)
Morphine (UV detection)	0.05	0.052	6.4	9.7
	0.50	0.497	3.8	6.9
	1.00	0.985	3.6	3.2
Morphine (Coulometric detection)	0.006	0.005	6.7	11.8
	0.05	0.048	4.6	8.0
	0.50	0.490	4.3	6.9
	1.0	0.994	4.2	7.5
Norcodeine	0.10	0.104	7.0	9.3
	0.80	0.787	3.5	5.6
	2.00	1.992	4.1	5.5
Codeine	1.00	1.059	7.2	8.4
	10.00	10.067	3.7	5.1
	50.00	49.329	4.6	4.6

significant peak tailing made the column unsuitable.

The Hypersil CPS column was chosen because it gave good resolution of all the compounds, with excellent peak shape, within a reasonable length of time, as shown by the chromatogram of a standard solution in Fig. 2a. These conditions also permit the simultaneous determination of the metabolites of morphine (retention times; morphine-3-glucuronide at 5.3 min, morphine-6-glucuronide at 5.9 min, and normorphine at 9.2 min).

3.2. Extraction

The extraction method was based on an existing procedure developed in our laboratory, for the extraction of morphine and its metabolites from plasma [17]. Four compounds were tested for use as an internal standard: nalorphine, dihydroxybenzylamine, levorphanol and DHCZ. Nalorphine was found to be unsuitable as an internal standard, because it co-eluted with the codeine, whilst dihydroxybenzylamine was not retained sufficiently on the solid-phase extraction

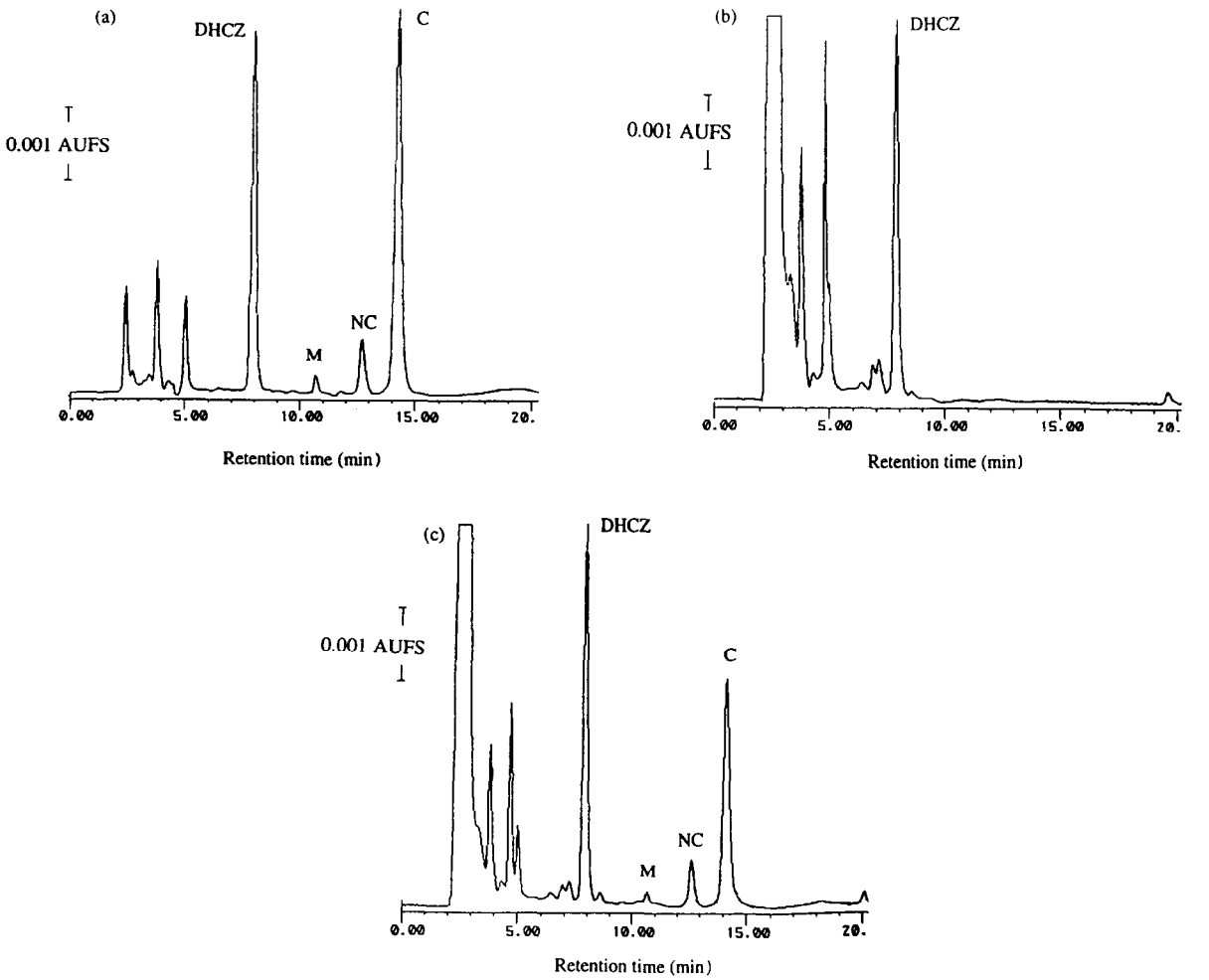

Fig. 2. Chromatograms of (a) calibration standard of 0.2 μg/ml morphine [M], 0.5 μg/ml of DHCZ, 0.5 μg/ml norcodeine [NC], and 5 μg/ml codeine [C]; (b) blank microsomal sample with 0.5 μg/ml of DHCZ; (c) microsomal sample incubated with 19.72 μg/ml codeine, containing 0.093 μg/ml of morphine, 0.5 μg/ml of DHCZ, 0.456 μg/ml of norcodeine and 3.10 μg/ml of codeine.

cartridge, and levorphanol had a very poor peak shape. DHCZ had an ideal retention time, eluting between morphine and norcodeine on the HPLC system, and gave a high and reproducible recovery when extracted with a C_{18} solid-phase cartridge. Under these conditions the recoveries expressed as the mean (± coefficient of variation), of the compounds, were found to be as follows: morphine 92.4% (±3.6%), DHCZ 87.1% (±6.8%), norcodeine 90.4% (±4.1%), and codeine 95.7% (±1.2%), taken as a mean of ten replicates.

3.3. Validation

Calibration curves showed good linearity between peak-area ratios and concentrations. Linear regression equations for the morphine, norcodeine and codeine standard curves were: $y = 8.47 \cdot 10^{-3}x - 8.05 \cdot 10^{-3} (r = 0.999)$, $y = 4.37 \cdot 10^{-4}x - 17.33 \cdot 10^{-3} (r = 0.998)$, and $y = 2.96 \cdot 10^{-1}x - 11.2 \cdot 10^{-3} (r = 0.999)$. The intra- and inter-assay precision was consistent over a wide concentration range, with coefficient of variation values being less than 10% (Table 1). Limits of detection were defined as three times the signal-to-noise ratio, and were found to be as follows: 2 ng/ml for morphine (by employing coulometric detection, this could be reduced to 250 pg/ml), and 5 ng/ml for both norcodeine and codeine.

3.4. Assay application

The N- and O-demethylation of codeine *in vitro* was studied by the incubation of codeine with liver microsomes, in the presence of an NADPH regenerating system, using a method based on that of Mikus *et al.* [18]. For the kinetic experiments 0.32 to 316 μg/ml codeine was incubated for 15 min. The microsomal samples were then analyzed to determine the amounts of morphine and norcodeine formed, from which the rates of O-demethylation and N-demethylation, respectively, could be calculated. Fig. 2b shows an example of a chromatogram of a blank microsomal sample, whilst Fig. 2c shows a chromatogram of a microsomal sample which had been incubated with 19.72 μg/ml codeine.

In summary, the procedure described, with its rapidity of sample preparation and the small sample volume required, offers significant advantages over existing HPLC methods for the determination of codeine and its metabolites.

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