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Note

Determination of codeine and its metabolites in human blood plasma and in microsomal incubates by high-performance liquid chromatography with ultraviolet detection

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A method has been developed for the determination of codeine and its metabolites, norcodeine, codeine-6-glucuronide (C6G) and morphine for use in kinetic studies of the drug in human plasma and liver microsomes. The method allows analysis of all four substances in a simple procedure. An alternative method, optimal for codeine and its metabolite norcodeine, is also described.

Analytical methods have been described for codeine and its metabolite morphine by radioimmunassay (RIA) [1–3] and gas chromatography–mass spectrometry (GC–MS) [4] and for morphine and its metabolites morphine-3- and -6-glucuronide and normorphine [5] by high-performance liquid chromatography (HPLC). This method has been extended by the introduction of an electrochemical detector in combination with a UV detector [6] in order to improve the detectability of morphine. This seems to be the method of choice when codeine, morphine and all the major metabolites are to be assayed in plasma and/or urine by a single procedure. However, this method is compli-

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cated and tedious, owing both to the solid-phase extraction step and to the combined detector approach. Because of this a simpler approach was investigated to fit our requirements. We expected the HPLC–UV method [5] for morphine to be useful also for codeine and its metabolites C6G and norcodeine. We found that this was the case for high dose levels, but for lower levels this method was not sensitive enough. After changing the extraction procedure and dividing it into two steps, we optimized the extraction to give high recovery and low limits of detection, especially for morphine and norcodeine. With a solvent extraction process these criteria were fulfilled except for C6G. This metabolite is too water-soluble to be extracted together with the non-conjugated compounds. For this reason C6G must be extracted using an adaptation of the Sep-Pak C_{18} cartridge methodology for morphine and its metabolites [5]. A detection level of 10 ng/ml for C6G was obtained, which was sensitive enough for measurement of this metabolite in plasma after oral and intravenous doses of codeine.

EXPERIMENTAL

Chemicals

Solvents were of commercial HPLC quality. Codeine was a gift from ACO (Stockholm, Sweden). Morphine chloride and codeine phosphate were obtained from the hospital pharmacy (Nord. Pharmacop.). Norcodeine was demethylated from codeine phosphate [7]. C6G was a gift from NIDA (Bethesda, MD, U.S.A.). NADP, glucose-6-phosphate dehydrogenase (G6PD) and glucose-6-phosphate were purchased from Sigma (St. Louis, MO, U.S.A.).

Instrumentation

The liquid chromatographic system consisted of a 2150 pump, a 7125 Rheodyne injector and a variable-wavelength UV detector (LKB, Bromma, Sweden), operated at a wavelength of 214 nm.

The column (150 mm × 4.6 mm I.D.) was slurry-packed with Nucleosil C_{18}, 5 μm particle size (Macherey-Nagel, Düren, F.R.G.), and the eluent consisted of acetonitrile (29%, v/v) in a mixture of 10 m*M* phosphate buffer (pH 2.1) and the ion-pairing agent, 1 m*M* sodium dodecyl sulphate. An alternative eluent, used for analysis of codeine and norcodeine, was acetonitrile (7%, v/v) in 10 m*M* phosphate buffer (pH 2.1), containing 2 m*M* propyl sulphate as the counter-ion.

Incubation

The oxidative formation of norcodeine and morphine in vitro was studied by incubation of codeine with liver microsomes in the presence of an NADPH-generating system. Liver microsomes were prepared according to ref. 8. The incubation mixtures (final volume 0.1 ml) contained 0.15 *M* Tris–HCl buffer

(pH 7.4), 5 m*M* magnesium chloride, 55 μM NADP, 2.5 m*M* glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase, 100 μg of microsomal protein and the substrate, 5 m*M* codeine. The reaction was started by addition of the NADP, and after incubation for 30 min at 37 °C it was stopped by freezing the tubes in acetone and dry ice. The tubes were stored at −70 °C until analysis.

Plasma assay

Codeine, norcodeine and morphine. Plasma (1 ml) was mixed with 1 ml of 1 *M* carbonate buffer (pH 10) and extracted with 4 ml of dichloromethane containing 15% (v/v) 2-propanol (shake board 10 min). The mixture was centrifuged (10 min at 500 *g*), after which the organic phase was transferred to a new tube and the solvent was evaporated under a stream of nitrogen at 40 °C. The residue was reconstituted in 300 μl of the eluent mixture. An aliquot (100 μl) was injected into the liquid chromatograph.

The same procedure was used when only codeine and norcodeine were analysed using the alternative eluent mixture (see above).

Codeine-6-glucuronide. Plasma (1 ml) was mixed with 3 ml of 0.5 *M* ammonium sulphate adjusted to pH 9.3 with aqueous ammonia (25%, v/v). The mixture was passed through a Sep-Pak C_{18} cartridge (Millipore Waters, Milford, MA, U.S.A.), which was then washed with 20 ml of 5 m*M* ammonium sulphate solution (pH 9.3 with ammonium hydroxide) followed by 0.5 ml of distilled water. The C6G was eluted from the cartridge with 3 ml of 28% (v/v) acetonitrile in 10 m*M* phosphate buffer (pH 2.1). An aliquot (100 μl) of this solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Extraction

A solvent extraction was used for codeine and its metabolites morphine and norcodeine. A pH of 10 was used during the extraction for optimizing the recovery of codeine and norcodeine. The extraction of morphine also gave an acceptable recovery at this pH. A detection limit of 1 ng/ml at a signal-to-noise ratio of 2:1 was obtained. The more water-soluble C6G could be analysed in the same system after extraction by the Sep-Pak C_{18} cartridge methodology. The detection limit for C6G with this method was higher (10 ng/ml) but sensitive enough for our studies of codeine kinetics after single doses or in vitro in liver microsomal preparations.

Liquid chromatography

This HPLC method is simple and selective for analysing codeine and its metabolites. Acetonitrile (29%, v/v) in a mixture of 10 m*M* phosphate buffer (pH 2.1) and 1 m*M* dodecyl sulphate was found to be a suitable solvent system for all substances. Norcodeine and codeine had retention times twice as long

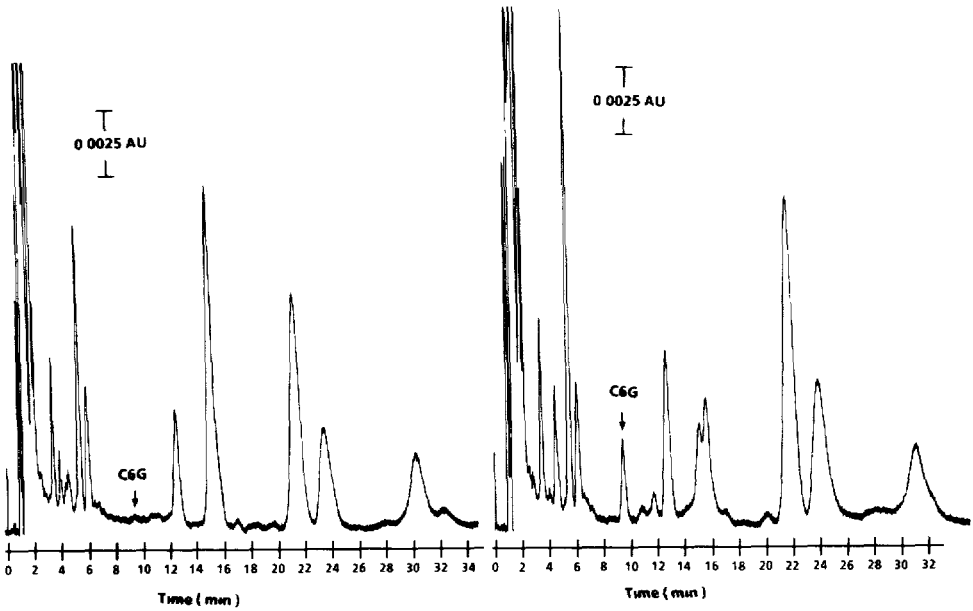

Fig. 1. Chromatograms obtained from (left) blank and (right) drug-free plasma sample spiked with codeine-6-glucuronide (250 ng/ml), morphine (5 ng/ml), norcodeine (25 ng/ml) and codeine (100 ng/ml). The sample was extracted following isolation on a Sep-Pak C_{18} cartridge. The peak for C6G was eluted after 9.4 min. Eluent, 29% (v/v) acetonitrile–10 m*M* phosphate buffer (pH 2.1)–1 m*M* sodium dodecyl sulphate.

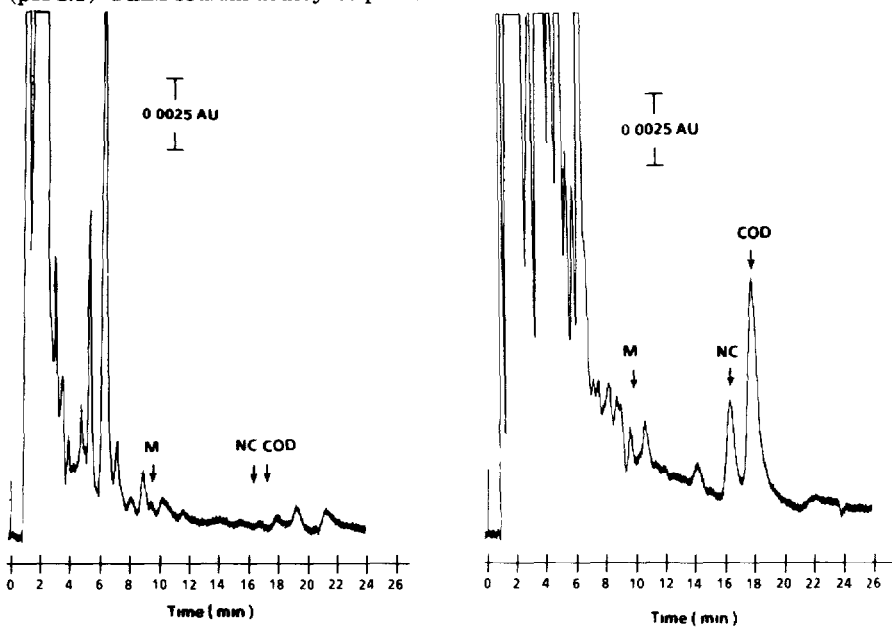

Fig. 2. Chromatograms obtained from (left) blank and (right) drug-free plasma sample spiked with 25 ng/ml morphine (M), 50 ng/ml norcodeine (NC) and 100 ng/ml codeine (COD). The sample was extracted with a solvent. The peaks for morphine, norcodeine and codeine were eluted after 9.4, 16.2 and 17.6 min, respectively, with the eluent used for Fig. 1.

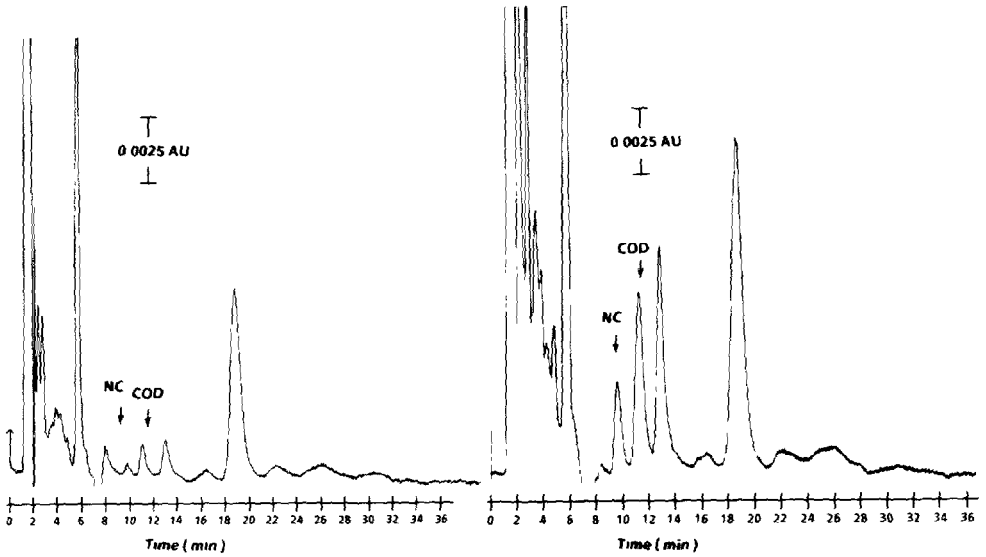

Fig. 3. Chromatograms obtained from (left) blank and (right) plasma sample spiked with 25 ng/ml morphine, 25 ng/ml norcodeine (NC) and 50 ng/ml codeine (COD). The sample was extracted with a solvent. The peaks for norcodeine and codeine were eluted after 9.4 and 11.2 min, respectively. Eluent, 7% (v/v) acetonitrile–10 m*M* phosphate buffer (pH 2.1)–2 m*M* propyl sulphate.

as those of C6G and morphine. Therefore, if only norcodeine and codeine compounds were to be analysed it was possible to employ an eluent containing acetonitrile (7%, v/v) in a mixture of 10 m*M* phosphate buffer (pH 2.1) and 2 m*M* propyl sulphate as counter-ion, which resulted in shorter retention times. Typical chromatograms are shown in Figs. 1–4. In chromatograms originating from some plasma samples, a background peak will appear with the same retention time as codeine (Fig. 3). In cases like this we have found that the detection limit of codeine might increase to 7 ng/ml.

To be able to analyse glucuronides and to get a suitable retention on the chromatographic column it is usually necessary to let them interact with an ion-pairing agent. A consequence of this technique is that polar endogenous compounds capable of forming ion pairs will be retained as well, and background peaks are frequently seen (Fig. 1). On the other hand, no plasma background in the area of the C6G peak was found.

Calibration

Drug-free plasma samples were spiked with morphine, norcodeine and codeine and analysed according to the method. The resulting peak heights were plotted versus the concentrations of the respective substances. Calibration was linear, but owing to the sample background variation the line did not always pass through the origin. With this in mind the determination limits had to be

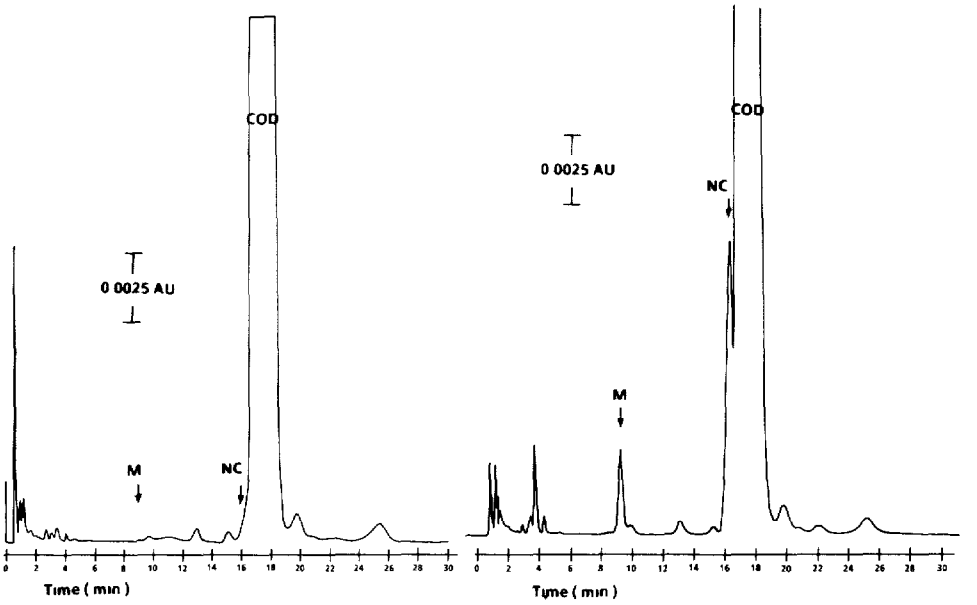

Fig. 4. (left) Blank sample chromatogram; (right) chromatogram depicting the formation of morphine (M) and norcodeine (NC) from codeine (COD) by human liver microsomes: morphine (O-demethylation) and norcodeine (N-demethylation) had retention times of 9.2 and 16.4 min, respectively, with the eluent used for Fig. 1.

adjusted according to the background variation in the sample. The standard curves constructed for the determination of C6G in plasma samples, and for the substances described above when used in microsome incubations, were linear and passed through the origin.

Capability of the method

The sensitivity, recovery and limits of detection of the present method for codeine and its metabolites are shown in Table I.

Application of the method

Our method was employed to study the single-dose kinetics of codeine postoperatively in cholecystectomized patients after an oral or intravenous dose. The latter route of administration yielded lower metabolite levels than the former. The elimination of codeine and the appearance of C6G, norcodeine and morphine are depicted in Fig. 5 for a representative patient.

A chromatogram obtained after an in vitro application of codeine metabolism in a human liver microsomal preparation is shown in Fig. 4. Velocity versus substrate concentration curves for codeine in human liver microsomes are illustrated in Fig. 6.

TABLE I

SENSITIVITY AND INTRA-ASSAY PRECISION OF THE HPLC ASSAY FOR CODEINE AND ITS METABOLITES IN HUMAN PLASMA SAMPLES

Compound	Concentration (ng/ml)	Coefficient of variation (%)	n	Recovery (%)	Detection limit (ng/ml)
Codeine	25	2.6	8	90	1
	100	7	8		
C6G	25	20.2	9	87	10
	250	14	10		
Morphine	2	10	10	83	1
	5	18.6	8		
Norcodeine	5	8.4	8	90	1
	25	7.6	8		

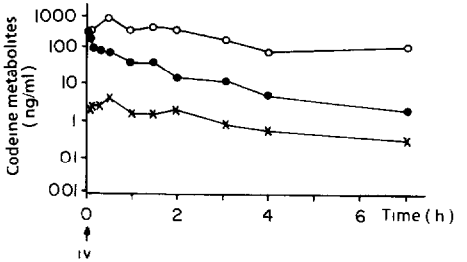

Fig. 5. Disposition of codeine and its metabolites in a patient given codeine as a single intravenous dose of 20 mg. (○) C6G; (●) codeine; (×) norcodeine.

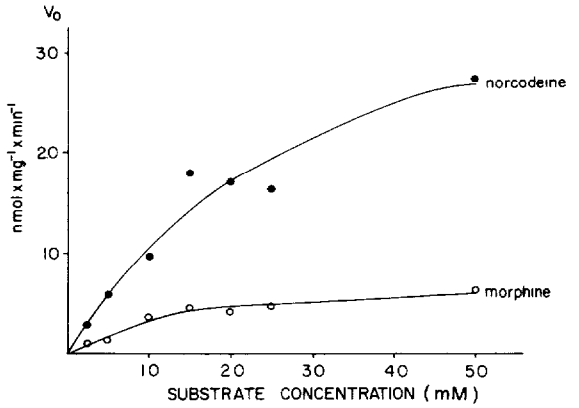

Fig. 6. Velocity versus substrate concentration curves with codeine in human liver microsomes.

ACKNOWLEDGEMENTS

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