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Simultaneous determination of dihydrocodeine and its metabolites in dog plasma by high-performance liquid chromatography with electrochemical and ultraviolet detection

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Abstract

An HPLC method with electrochemical and UV detection was established for the simultaneous determination of dihydrocodeine and its metabolites, dihydronorcodeine, dihydromorphine, and dihydrocodeine glucuronide, in dog plasma using N-ethylnormorphine as the internal standard. The method involved sample pretreatment with a C_{18}-bonded disposable column, and the injected fraction was separated and detected on the C_{18}-bonded column with serially coupled UV and coulometric detectors. Dihydromorphine was detected with the coulometric detector at 0.4 V, and dihydrocodeine and dihydronorcodeine at 0.8 V. Dihydrocodeine glucuronide was detected with UV at 210 nm. Recoveries of the studied compounds were quantitative at the individual assay ranges, and validation of the assay gave results that were satisfactory in terms of within-run or between-run precision and accuracy. Lower limits of quantitation were 2 ng/ml for dihydrocodeine and dihydronorcodeine, 0.5 ng/ml for dihydromorphine, and 200 ng/ml for dihydrocodeine glucuronide.

1. Introduction

Dihydrocodeine (DHC) is widely used as an antitussive and analgesic drug. Due to its moderate potency as an analgesic, DHC has been nominated for use as a second-step drugs in therapeutic strategy, a three-step analgesic ladder , recommended by the World Health Organization for cancer pain relief [1]. DHC is used in a variety of formulations, but the controlled-release type should be the first choice for cancer patients, considering the success of controlled-released morphine. For determination of DHC several methods have been reported using GC [2], HPLC [3–6], and immunological assay [7]. However, none has described a method for simultaneous assay of DHC and its metabolites, due to limited knowledge of DHC metabolism in humans and animals. We previously characterized the metabolism and pharmacokinetics of DHC in dogs, in which the major metabolic pathway of DHC was found to be analogous to the codeine metabolism [8]. The metabolites found in dog urine were dihydronorcodeine (DHNC), dihydromorphine (DHM), dihydrocodeine-6-glucuronide (DHCG), and dihydronorcodeine-6-glucuronide (DHNCG).

This paper describes a sensitive assay for DHC and its metabolites, DHNC, DHM, and DHCG, in dog plasma by HPLC with coulometric and

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UV detection. The method was successfully applied for the measurement of plasma concentrations of these drugs after oral administration of DHC to dogs. The usefulness of the method was proved for pharmacokinetic analysis of DHC and its metabolites.

2. Experimental

2.1. Materials

Reagent grade sodium dihydrogen phosphate dihydrate and ammonium sulfate were obtained from Nacalai Tesque (Kyoto, Japan), 85% phosphoric acid solution and sodium dodecylsulfate (SDS) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 28% ammonia was obtained from Yoneyama Pharmaceuticals (Osaka, Japan). HPLC-grade methanol and acetonitrile were purchased from Kanto Chemical (Tokyo, Japan). Water was purified before use with ultra-pure water generating equipment (Barnstead, Boston, MA, USA). DHC phosphate was obtained from Shionogi (Osaka, Japan), and N-ethylnormorphine (ENM) was purchased from Laboratories Sarget (Merignac Cedex, France). DHM hydrochloride, DHNC hydrochloride, and DHCG of more than 99% purity were synthesized by the procedure described elsewhere [8].

2.2. Preparation of buffer solutions

For the preparation of phosphate buffer solution containing 1.5 m*M* of SDS (pH 2.4), 1.3 g of SDS and 23.4 g of sodium dihydrogen phosphate dihydrate were dissolved in *ca.* 2500 ml of water. The pH of the solution was adjusted to 2.4 by adding 85% phosphoric acid drop-wise, and making up to 3000 ml with water.

For 0.5 *M* ammonium buffer solution, dissolve 33 g of ammonium sulfate in *ca.* 400 ml of water, adjust the pH of the solution to 9.6 by adding 28% ammonia dropwise, and make up to 500 ml with water. Prepare a 5 m*M* ammonium buffer solution (pH 9.6) by dissolving 0.6 g of ammonium sulfate in *ca.* 800 ml of water and make up to 1000 ml with water in a manner similar to that described above.

2.3. Instrumentation

The flow diagram used in this study is shown in Fig. 1. A Hitachi L-6000 pump (Hitachi, Ibaragi, Japan) was used to deliver the mobile phase. Detectors used were an ESA Coulochem 5010A (Bedford, MA, USA) operating with an ESA 5100A dual-electrode cell set at an oxidation voltage of 0.4 V for the first electrode and 0.8 V for the second electrode, and a Shimadzu SPD-6AV spectrophotometer (Kyoto, Japan) with pressure-resistant flow cell (path length, 6 mm; cell volume, 3 μl) operating at a wavelength of 210 nm. The signal outputs from the first electrode were smoothed with a Signal Cleaner (Model SC-77, System Instruments, Hachioji, Tokyo, Japan). The conditioning cell, an ESA Model 5021 controlled at 0.15 V with a potentiostat (Model NPOT-2501, Nikko Keisoku, Atsugi, Kanagawa, Japan), was placed between the spectrophotometer and the coulometric detector to preoxidize the eluates from the UV detector. The mobile phase was oxidized to remove impurities with an ESA 5020 guard cell placed in the outlet of the pump, operating at 0.85 V. Samples were injected via a Waters WISP 710B Sample Processor (Millipore, Milford, MA, USA) using cooling units. Two

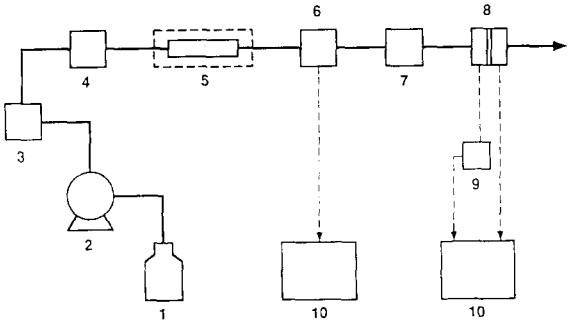

Fig. 1. Schematic flow diagram of the HPLC system. (1) Mobile phase reservoir; (2) pump; (3) guard cell; (4) autosampler; (5) analytical column with column heater; (6) UV detector; (7) conditioning cell; (8) analytical cell; (9) signal cleaner; (10) data processor.

Shimadzu C-R4AX data processors were used for the individual detectors.

2.4. Chromatographic conditions

The analytical column was a 5-μm Crest-Pak C18S, 150 × 4.6 mm I.D. (Jasco, Hachioji, Tokyo, Japan). The mobile-phase composition was phosphate buffer (pH 2.4) containing 1.5 m*M* SDS–acetonitrile (3:1, v/v). The flow-rate was 1 ml/min. Column temperature was kept at 35°C with a column heater (Scientific System Model CH-20, State College, PA, USA).

2.5. Preparation of standard solutions

Dissolve 1 mg each of DHC, DHNC, DHM, and 5 mg of DHCG as a free base in 10 ml water to make up stock solutions. Prepare standard solutions of five concentrations by dilution of the stock solutions with water, containing 0.08, 0.2, 0.4, 1, 2 μg/ml of DHC and DHNC, 0.02, 0.05, 0.1, 0.25, 0.5 μg/ml of DHM, and 8, 20, 40, 100, 200 μg/ml of DHCG. Dissolve and dilute 1 mg of ENM with water to make up the standard solution of the internal standard containing 10 μg/ml of ENM. Use a 25-μl aliquot of each solution to prepare a calibration curve.

2.6. Assay procedure

To a Sep-Pak C_{18} cartridge (Waters, packing weight: 360 mg) with a 5-ml disposable syringe (Terumo, Tokyo, Japan) which had been pre-washed successively with 10 ml of methanol, 5 ml of pH 2.4 phosphate buffer containing 20% of acetonitrile, and 10 ml of water, were applied a solution consisting of 1 ml of sample plasma, 25 μl of water, 25 μl of ENM solution, and 3 ml of 0.5 *M* ammonium buffer (pH 9.6) to adsorb on the column with aspiration *in vacuo*. The column was washed with 20 ml of 5 m*M* ammonium buffer (pH 9.6) and 20 ml of water; then the fraction of interest was eluted with 2 ml of pH 2.4 phosphate buffer containing 20% of acetonitrile. A 200-μl aliquot of the eluted test solution was injected onto the HPLC column.

2.7. Calibration curve

To 1 ml of drug-free plasma were added 25 μl of standard solutions of five known concentrations, 25 μl of ENM solution, and 3 ml of 0.5 *M* ammonium buffer (pH 9.6). The mixture was applied to a Sep-Pak C_{18} cartridge followed by the same purification as in the assay procedure. The calibration curve was prepared by plotting the peak-height ratio of the individual compounds to ENM against the added concentrations. The calibration curve was prepared daily on the day of assay.

2.8. Recovery

To 1 ml of drug-free plasma were added 25 μl of aqueous solutions of known concentrations of DHC, DHNC, DHM and DHCG, and 25 μl of the ENM standard solution, and the mixture was treated in the same manner as described in the assay procedure. The calibration curve was prepared with an aqueous solution containing the same ranges of standard concentration.

2.9. Stability tests

Aqueous solution

Solutions containing 1 μg/ml each of DHC, DHNC, DHM, DHCG, and ENM were stored at 4°C and −20°C, and 200-μl aliquots of the solutions were used for HPLC for up to 4 months.

Test solution

Drug-free plasma spiked with the drugs containing 50 ng/ml each of DHC and DHNC, 13 ng/ml of DHM, and 5 μg/ml of DHCG, was treated according to the assay procedure, then the eluted test solution was assayed on the day of the treatment and after 3 weeks preservation at 4°C.

Plasma

Drug-free plasma spiked with the drugs containing 50 ng/ml each of DHC and DHNC, 13 ng/ml of DHM, and 5 μg/ml of DHCG was

stored at −20°C, and the samples were assayed for up to 4 months.

2.10. Assay performance

Three pooled plasma samples with high, medium, and low concentrations of the drugs were prepared containing 2, 10, 50 ng/ml of DHC and DHNC, 0.5, 2.5, 13 ng/ml of DHM, and 200, 1000, 5000 ng/ml of DHCG. Within-run precision and accuracy were evaluated by five repeated assays of each pooled plasma according to the assay procedure. Between-run precision and accuracy were estimated by assay of each pooled plasma daily for five days.

2.11. Animal protocol

DHC phosphate was administered orally as a 20-ml aqueous solution via rubber tubing to three fasted beagle dogs at a dose of 1 mg/kg. A 5-ml sample of heparinized blood was drawn at 0.5, 1, 2, 3, 5, 7, and 10 h after administration. Plasma was immediately separated from blood, frozen and stored at −20°C until assay.

3. Results and discussion

3.1. Hydrodynamic voltammograms

Fig. 2 shows the hydrodynamic voltammograms of DHC, DHNC, DHM, DHCG, and ENM. The peak responses from the coulometric detector were measured for each 30-pmol injection of these compounds under the assay conditions. Both DHM and ENM began to be oxidized at 0.2 V and their responses reached a plateau at 0.3 V. The oxidative responses of these two compounds were thought to be based on the oxidation of the phenolic 3-hydroxyl group in their structures as was found for the morphine molecule [9,10]. In contrast, for DHC, DHNC, and DHCG, oxidation did not occur up to 0.5 V, while weak responses were found at 0.6 V. The optimal responses were obtained at 0.8 V and were attributed the oxidation of the anisole moiety in their structures. The results enabled us to distinguish these structure-related molecules by detection with differential oxidation voltages using dual electrodes.

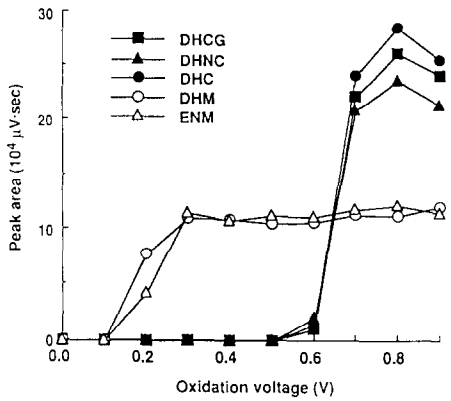

Fig. 2. Hydrodynamic voltammograms of DHC and related compounds under assay condition. (●) DHC; (▲) DHNC; (■) DHCG; (○) DHM; (△) ENM.

3.2. Prechromatographic treatment and assay specificity

Prechromatographic treatment for the determination of DHC, DHNC, DHM, and DHCG in dog plasma was done with a disposable column for solid-phase extraction. The procedure has previously been described [9,11,12]. The optimal procedure was obtained with a Sep-Pak C_{18} cartridge using pH 9.6 buffer and pH 2.4 buffer for the adsorption and the elution solutions, respectively. For separation, the optimal result was obtained with a CrestPak C18S as the analytical column using a mobile phase consisting of pH 2.4 buffer containing 1.5 m*M* SDS–acetonitrile (3:1, v/v). Detection was done with the coulometric detector at an oxidation voltage of 0.4 V for DHM and ENM and of 0.8 V for DHC and DHNC. Detection by UV spectrophotometry was employed for DHCG analysis because the linearity of the coulometric response was not satisfactory due to its higher concentration range. Furthermore, the calibration curve showed a nonlinear response for the amounts injected due to the concentration-dependent oxidation by the coulometric detector when the UV spectrophotometer was placed after the

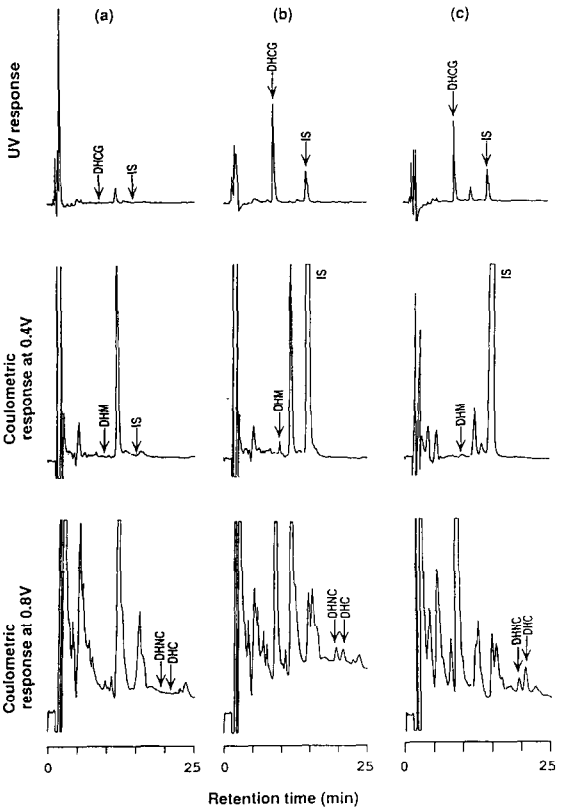

Fig. 3. Typical chromatograms of blank plasma (a), blank plasma spiked with drugs (DHC, 10.4 ng/ml; DHNC, 11.6 ng/ml; DHM, 2.6 ng/ml; DHCG, 981 ng/ml) and internal standard (250 ng/ml) (b), and sample plasma (DHC, 14.6 ng/ml; DHNC, 11.7 ng/ml; DHCG, 549 ng/ml) after administration of DHC (c). Upper, UV detection; middle, coulometric detection at 0.4 V; bottom, coulometric detection at 0.8 V.

coulometric detector. This problem was solved by placing the UV detector before the coulometric detector. Fig. 3 shows the chromatograms obtained by this assay method. No components in dog plasma interfered with this assay. As a result, a comparably simple procedure was established to determine DHC and its metabolites in dog plasma.

3.3. Calibration curve and linearity

Table 1 shows the parameters of the calibration curve obtained from the daily calibration curves prepared in each assay. The assay concentration ranges established were 2–50 ng/ml for DHC and DHNC, 0.5–13 ng/ml for DHM, and 200–5000 ng/ml for DHCG, respectively. The R.S.D.s of the regression curve were $<6\%$ and the averages of the coefficients of correlation were >0.999 for the compounds tested.

3.4. Recovery from dog plasma

Analytical recoveries of the compounds from dog plasma were 103% for DHC, 101% for DHNC, 96% for DHM, and 93% for DHCG at the individual assay ranges. Absolute recoveries were found to be 93–105% for these drugs. The results indicated satisfactory recovery in this assay.

3.5. Assay performance

The results of the assay performance are shown in Tables 2 and 3. In the within-run assay, precisions represented as R.S.D.s were $<8.1\%$ and accuracies estimated as the percents of bias to the theoretical concentrations were between -10.2% and $+12.7\%$ for the drugs tested. The same order of performance was found for these

Table 1
Linear least-square regression analysis of calibration curves for DHC and its metabolites ($n = 17$)

Compound	Concentration range (ng/ml)	Slope		y-Intercept	Regression coefficient	
		Mean	R.S.D. (%)		Mean	R.S.D. (%)
DHC	2–50	0.0858	6.3	0.00536	0.999	0.04
DHNC	2–50	0.0740	5.4	0.00400	0.999	0.02
DHM	0.5–13	0.00510	5.8	0.000295	0.999	0.01
DHGC	200–5000	0.00436	4.9	0.000215	0.999	0.01

Table 2
Within-run precision and accuracy for the determination of DHC and its metabolites ($n = 5$)

Compound	Concentration added (ng/ml)	Concentration measured (ng/ml)	Precision (R.S.D., %)	Accuracy (bias, %)
DHC	2.07	1.86	7.2	−10.2
	10.4	10.7	2.9	+3.0
	51.8	53.5	2.0	+3.4
DHNC	2.32	2.13	7.4	−8.1
	11.6	11.5	6.4	−0.7
	58.0	57.9	2.8	−0.1
DHM	0.525	0.592	8.1	+12.7
	2.62	2.59	2.7	−1.3
	13.1	12.8	1.3	−2.7
DHCG	196	189	1.7	−3.5
	981	978	1.1	−0.4
	4910	5160	3.5	+5.1

compounds in the between-run assay. The results were found to satisfy the criteria of the analytical method validation [13].

3.6. Drug stability

In aqueous solutions DHM, DHCG, and ENM were stable at least for 4 months after storage at 4°C and −20°C, while DHC and DHNC were stable up to 3 months at these temperatures. In the test solutions DHC, DHNC, DHM, and DHCG were stable for at least 3 weeks at 4°C. In plasma DHC, DHNC, DHM, and DHCG were stable for at least 4 months on storage at −20°C.

3.7. Application of the method

The method was used to measure the concentrations of DHC and its metabolites in dog plasma after DHC administration. Fig. 4 shows the average plasma concentrations of DHC, DHNC, and DHCG after a 1 mg/kg oral administration of DHC phosphate as aqueous solutions to two beagle dogs. The peak plasma concentration of DHC was found to be 12.9 ng/ml at 0.5 h after administration, then DHC

Table 3
Between-run precision and accuracy for the determination of DHC and its metabolites ($n = 5$)

Compound	Concentration added (ng/ml)	Concentration measured (ng/ml)	Precision (R.S.D., %)	Accuracy (bias, %)
DHC	2.07	2.27	9.6	+9.9
	10.4	10.7	10.4	+3.3
	51.8	52.9	2.0	+2.2
DHNC	2.32	2.26	10.3	−2.9
	11.6	11.3	4.8	−2.8
	58.0	56.7	2.1	−2.3
DHM	0.525	0.508	8.5	−3.2
	2.62	2.54	6.4	−3.4
	13.1	12.5	3.2	−4.9
DHCG	196	192	1.7	−2.2
	981	941	2.7	−4.1
	4910	5280	2.9	+7.7

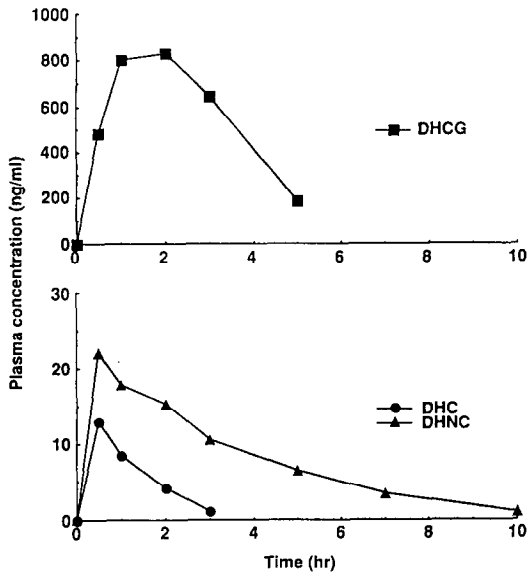

Fig. 4. Plasma concentration–time curves for DHC, DHNC, and DHCG after 1 mg/kg oral administration of aqueous DHC phosphate solution to two dogs.

rapidly disappeared from plasma. The DHC concentration decreased to less than 2 ng/ml at 5 h after administration. The average DHNC concentration reached a maximum of 22.1 ng/ml at 0.5 h, then gradually decreased to 1.1 ng/ml (2.2 ng/ml and <2 ng/ml for the two dogs) at 10 h after administration. DHCG showed a peak concentration of 828 ng/ml at 2 h after administration and disappeared from plasma to the concentration of less than 0.2 μg/ml at 7 h after administration. These results indicate that DHCG is the major metabolite and significant concentrations of DHNC are observed in plasma after administration of DHC. Urinary excretions of DHC and its metabolites were found to be 1.6% for DHC, 3.0% for DHNC, 49% for DHCG, and 0.1% for DHM after a 3 mg/kg oral administration of DHC [8]. It was proven that the plasma profiles obtained in this study reflected the urinary excretions of DHC and its metabolites after administration of DHC. The lower plasma level of unchanged DHC is assumed to be due to a hepatic first-pass effect after oral administration. Plasma concentrations of DHM are less than the lower limit of quantification for the samples in this experiment. DHM is suggested to be metabolized to its glucuronide, similar to the process found in codeine metabolism [6,12,14–18].

Thus, the assay established in this work is satisfactory for use with plasma samples after administration of DHC to dogs.

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