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Short Communication

Automatic on-line extraction coupled with electrochemical detection as an improved method for the HPLC co-analysis of codeine and morphine in plasma and gastric juice*

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Introduction

Considerable difficulties were found when operating the standard high-performance liquid chromatography method for morphine and its metabolites [1]. It was suggested that the basis of these difficulties lies with the quality of clean-up of the extracts obtained from solid-phase extraction. This was evident from the fact that contamination of the electrochemical cell occurred gradually throughout each run. The serum components such as fibrin and polar lipid material adhere to the porous carbon surface of the electrode reducing diffusion of drug species to the electrode surface and increasing the flow of current by oxidation and subsequent reduction due to electron transfer from the solvent. This necessitated frequent clean-up of the electrodes with nitric acid and eventual replacement of the complete cell unit. Clearly a way of side-stepping the problems of contamination would be to reduce the content of faradic impurities in the sample by a more efficient extraction step.

The technique of on-line extraction offers several advantages in assay design [2]. One main advantage is that purification of samples is very efficient [3]. Another advantage is extremely good accuracy and precision [4, 5] and the ability to amplify peak response according to the theory of peak compression [6] when used in the backflush mode. A disadvantage of backflushing the pre-column is a loss of the filter effect of the pre-column [7]. This can be compensated for by the installation of an in-line 2 μm filter (Jones Chromatography, Hengoed). The testing of this method of filtering the sample was an important part of the study as one would expect a narrower peak shape and longer pre-column life to result. Another important consideration was the choice of packing material. A wide range of pre-column packing materials and methods have been utilized for on-line extraction [8]. The attributes of a good packing material are near quantitative recovery of parent drug, cheapness and ease of packing into pre-column cartridges by the assay chemist. A number of such materials were tested for compatibility in an assay involving codeine and morphine in serum and gastric juice.

The analysis of codeine in serum has been reported in many studies [9–11]. No work has so far been reported on the pharmacokinetics of codeine in the stomach. It has been reported that some basic drugs such as quinine, are partitioned into the acidic contents of the human stomach from the systemic circulation [12]. From this observation, it was suggested that a basic drug such as codeine would be

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similarly partitioned into gastric juice. The present study investigates an on-line extraction technique to test this hypothesis.

Experimental

Materials

Buffer chemicals were of Analar grade (BDH Ltd, Poole). Phosphate buffer (pH 7.4; 40 mM) was prepared from disodium hydrogen orthophosphate dihydrate (30.7 mM) and potassium dihydrogen orthophosphate (8.7 mM). Extractant material was selected from a range of standard stationary phase materials including Lichroprep RP-2 (BDH Ltd, Poole, Nucleosil 30 C18, and proprietary material for solid-phase extraction of narcotics including NARC-1 (J.T. Baker UK, Hayes) and CERTIFY (Jones Chromatography, Hengoed). Incubation buffer was prepared from anhydrous sodium acetate (2 M) adjusted to pH 6.0 with acetic acid LR (BDH Ltd, Poole). β-Glucuronidase, Type B-10, ex bovine liver (Sigma Chemical Co.) was dissolved in incubation buffer to make a stock solution of 10^8 units l^{-1} which was stored at 4°C for up to a week. Morphine-3β-D-glucuronide (Sigma Chemical Co.) was used as control substrate. Methanol was of HPLC purity (Rathbone Ltd, Walkerburn). All water was drawn from a Spectrum in-house laboratory still (Elga Ltd, High Wycombe).

Volunteer study

A healthy volunteer was intravenously infused with pentagastrin solution for 15 min followed by a single intramuscular dose of 0.3 mg kg^{-1} codeine phosphate solution (26 mg equivalent of the hemi-hydrated salt). Pentagastrin was continuously infused for a further 15 min. Total aspirates of gastric juice were withdrawn at half-hourly intervals via an indwelling naso-gastric catheter. The end of the tube containing the catheter and aspirate ports maintained a seal between the bile duct and the top of the duodenum by means of an inflatable balloon. The purpose of this device was to prevent back-diffusion of bile into the stomach and to keep samples essentially bile free. Blood samples (10 ml) were withdrawn at regular time intervals up to 8 h post-dose. The blood was separated into serum and red cells by centrifugation (2000*g* for 10 min) and samples frozen at −20°C pending further analysis.

Enzyme hydrolysis

Blood serum samples (1.0 ml) were mixed with incubation buffer (250 μl) and glucuronidase solution (50 μl). They were then sealed and heated for 15 h at 50°C. A control solution of 1000 μg l^{-1} morphine-3-glucuronide in blank serum was similarly treated together with a set of calibration standards. The total volume of hydrolysate was 1.3 ml.

Analysis of samples

The apparatus was constructed around a pneumatically actuated two-way, six-port column switching valve (Masterchrom Ltd, Slough; Fig. 1). Two pumps, a Shimadzu LC-6A extraction/wash pump (Dyson Instruments, Heaton) and a Kratos Spectroflow 400 elution pump (Severn Analytical, Shefford), were used for liquid handling. The system controller was a Gilson 231 autosampler (Anachem Ltd, Luton) which also controlled the injection of samples via a robotic arm. Sample transfer was the most satisfactory with 0.5 ml Eppindorf tubes. A system interface (Masterchrom Ltd, Slough) enabled the controller to communicate with the valve and the extraction/wash pump. Samples were extracted onto a Lichroprep RP-2 pre-column cartridge (2 × 0.46 cm i.d., 25–40 μm; BDH Ltd, Poole). The main column was a Spherisorb ODS-2 (15 × 0.46 cm i.d., 5 μm) cartridge column (Chrompak UK Ltd, London) heated to 50°C, to reduce peak broadening, in a column oven (Jones Chromatography, Hengoed). Drugs were eluted in methanol–phosphate buffer (pH 7.4; 40 mM; 40:60%, v/v) and detected electrochemically at an oxidizing voltage of +0.9 V, on a Coulochem 5510A/5011 porous graphite electrode (Severn Analytical, Shefford). Background current was suppressed by use of an on-line guard cell set at +0.95 V and positioned upline from the autosampler.

Samples for extraction were centrifuged (2000*g* for 10 min) and aliquots of the supernatant (approximately 0.4 ml) removed to Eppendorf tubes (0.5 ml capacity). Gastric juice was diluted (1:10, v/v) in phosphate buffer (pH 7.4; 40 mM) before removal. Extractant material which gave the best recovery of morphine and codeine (Lichroprep RP-2), was dry packed into pre-columns before each experiment. The packed pre-columns were conditioned with five blank samples followed by duplicate samples of the highest calibration standard, ending with a final blank sample.

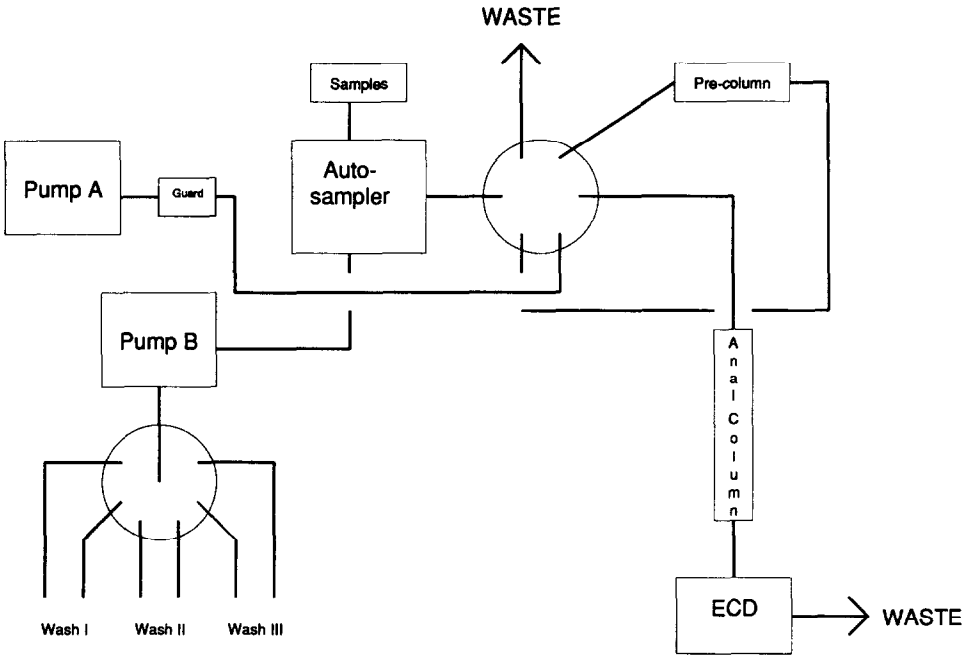

Figure 1
Pre-column switching apparatus used to assay morphine and codeine in plasma and gastric juice. The pre-column was a Lichroprep RP-2 (2 × 0.46 cm i.d., 25–40 μm) the analytical column was a Spherisorb ODS-2 (15 × 0.46 cm i.d., 5 μm). Mobile phase (Pump A) was methanol–phosphate buffer (pH 7.4; 40 mM; 40:60, v/v). Wash solvents (Pump B) were (i) methanol–water (5:95, v/v), (ii) mobile phase, (iii) methanol. Flow rate was 1.0 ml min^{-1}. Temperature was 50°C. Electrochemical detection voltage (differential) was + 0.9 V (Channel 1) + 0.4 V (Channel 2). Sample injection size was 100 μl.

Samples (100 μl) were injected by the total loop filling method. The first wash period or extraction time was 3 min at a flow rate of 2 ml min^{-1}. The wash solvent consisted of either methanol–disodium hydrogen phosphate (pH 8.5; 5 mM; 5:95%, v/v), used for plasma only, or methanol–water (5:95%, v/v) used for hydrolysed plasma and gastric juice. After this period the valve was switched, the detector zeroed and data capture begun. A backflush time of 24 s elapsed while extracted drug was eluted onto the analytical column at either 0.8 ml min^{-1} (plasma only) or 1.0 ml min^{-1}. Elution times were selected as appropriate for each sample type. A further delay period had to be used for gastric samples because of a late running peak. The cycle continued with a wash period of 70 s in methanol–phosphate buffer (pH 7.4; 40 mM; 40:60%, v/v), methanol and finally methanol–water (9:95%, v/v) which regenerated the pre-column for the next sample. The developed chromatogram was printed out, analysed and raw data stored on floppy disc for further reanalysis. All samples were run in duplicate. Quality control samples were interspersed within the array of samples in a computer generated randomized fashion with running standards included. Calibration was made at the start and end of each experiment. No internal standard was added. Typical chromatograms (Fig. 2) show peak shape and background levels for plasma and diluted gastric samples.

Results and Discussion

Volunteer study

The absorption of a single intravenous dose of codeine (26 mg; 0.3 mg kg^{-1}) gives a maximum measured concentration of 203.71 μg l^{-1} 15 min after the end of infusion (Table 1). From this point the codeine concentration in the circulation reduces until the limit of detection is reached after 8 h. Metabolism of parent drug occurs in the liver to give the glucuronide conjugate of codeine which appears in the blood reaching a maximum measured concentration of 381.98 μg l^{-1} after 2 h. This then reduces gradually by renal clearance. Demethylation of codeine to form

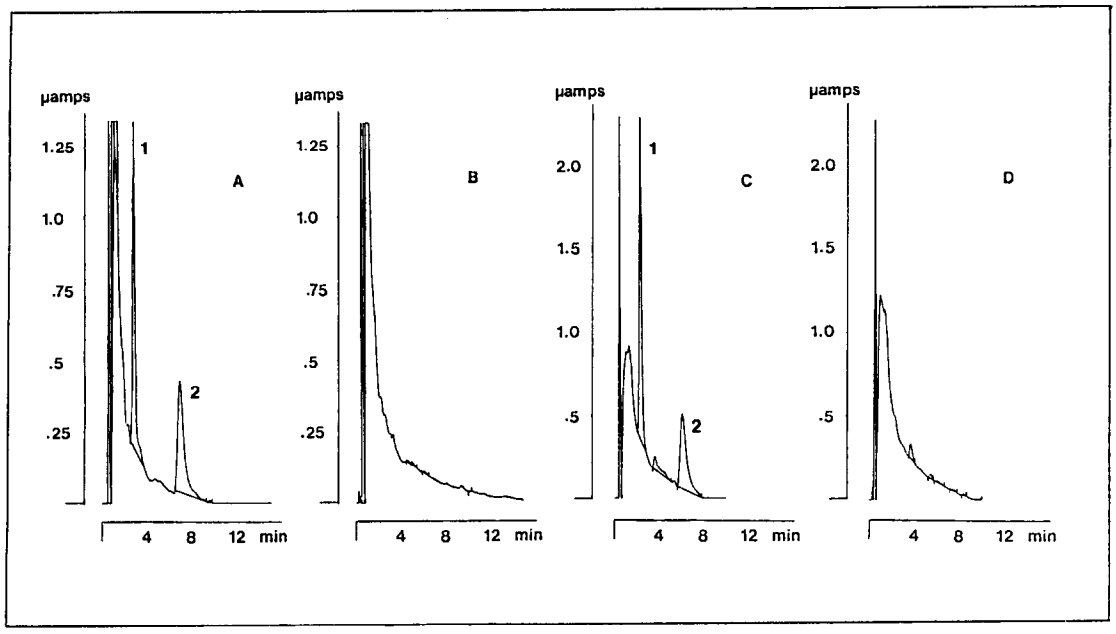

Figure 2
Chromatograms of morphine and codeine under typical assay conditions. Column was Spherisorb ODS-2 (15 × 0.46 cm i.d., 5 μm); flow rate 1.0 ml min^{-1}; temperature 50°C; mobile phase was methanol–phosphate buffer (pH 7.4; 40 mM; 40:60, v/v). Samples were extracted on-line from methanol–water (5:95, v/v) onto a Lichroprep RP-2 pre-column (2 × 0.46 cm i.d., 25–40 μm) at 2.0 ml min^{-1} and backflushed for 24 s at 1.0 ml min^{-1}. Morphine sulphate and codeine phosphate were spiked at 200 μg l^{-1}. Gastric juice was diluted (1:10) with phosphate buffer (pH 7.4; 40 mM). Samples are (a) spiked serum, (b) blank serum, (c) spiked diluted gastric juice and (d) blank diluted gastric juice; 1 = morphine; 2 = codeine.

Table 1
Concentrations of analyte following a 26-mg intramuscular dose of codeine phosphate to a healthy volunteer. Blood was centrifuged (2000*g* for 10 min) to separate plasma from red cells. Total aspirates of stomach contents were diluted (1:10) with phosphate buffer (pH 7.4; 40 mM). All results for stomach contents were corrected for dilution. Codeine glucuronide in serum (1.0 ml) was hydrolysed with β-glucuronidase, Type B-10, 10^8 units l^{-1} (50 μl) in sodium acetate buffer (pH 5.6; 2 M; 25 μl) at 50°C for 15 h. Codeine concentrations in hydrolysates were corrected by 0.815 to account for recovery

	Plasma			Stomach contents	
Time (h)	Codeine (μg l^{-1})	Codeine glucuronide (μg l^{-1})	Morphine (μg l^{-1})	Codeine (μg l^{-1})	Morphine (μg l^{-1})
0.25	203.41	24.44	0.48	—	—
0.5	203.71	135.2	1.04	147.5	ND
0.75	132.42	254.27	ND	—	—
1	111.46	345.89	1.53	1567.5	ND
1.5	99.39	338.27	1.83	2811	ND
2	70.23	381.98	ND	744.25	ND
2.5	58.38	363.85	ND	1103.3	17.36
3	51.84	324.48	ND	1291.2	ND
3.5	46.01	321.83	ND	599.65	36.6
4	38.35	302.25	ND	—	—
5	28.69	238.2	ND	—	—
6	17.33	201.98	ND	—	—
7	16.83	146.86	ND	—	—
8	13.81	122.02	ND	—	—

morphine is known to occur after oral dosing of codeine at a plasma concentration of about 2–3% during steady state [9]. The results show that there is no evidence to suggest that morphine is formed at a higher level than this following intravenous dosing and that the morphine probably is formed from the breakdown of codeine glucuronide rather than from parent drug. Caution must be exercised in interpreting the morphine data as very low

levels of morphine, below the estimated limit of detection are in evidence. The trend, however, is for a gradual increase in circulating morphine up to 3 h post-dose. Absence of data from later time points may be due to progressive broadening of the morphine peak.

The identification of the glucuronide conjugate of codeine as the 6-glucuronide is supported by previous work [13]. Metabolite profiles of codeine in human urine [14], indicate that codeine-6-glucuronide is the only metabolite of significance in man. As codeine-6-glucuronide was not available for testing, the choice of morphine-3-glucuronide as test compound depended on the assumption that recovery of morphine under assay conditions would be similar to that of codeine. A recovery of 81.5% (81.0; 82.1) was obtained with morphine-3-glucuronide. This correction was taken as the best estimate of codeine recovery after hydrolysis. Glucuronidase from bovine liver has been used in previous work on morphine and codeine [15] to quantify levels of the conjugates and shown to be satisfactory although recommendations have also been made for Type L-2 [16] and Type H-5 [17].

A concentration of codeine in the gastric lumen of nearly thirty times the level in plasma (after 1.5 h) (Table 1) is a surprising result and supports the hypothesis of pH trapping of basic compounds in the stomach. The concentration curve was biphasic with a second maximum at 25 times the plasma level (after 3 h). The trapping also seemed to occur for morphine although again, levels were lower than the estimated limit of detection allowing for correction of volume. This effect is of interest in understanding the distribution of basic narcotic drugs in the human body and is the subject of further study in our department.

Electrochemical detection

Many workers have reported using electrochemical detection in the analysis of morphine and codeine. The advantage of this method of detection arises from the sensitivity of phenolic 3-hydroxy and tertiary amine groups in the structures of these compounds. Both groups are oxidized between +0.6 and +1.2 V. Oxidation of the 3-hydroxy group has the lower electrochemical potential. The response curves for morphine and codeine at acidic pH are given as smooth curves (Fig. 3). Smooth curves for this voltametric oxidation have been confirmed in previous studies [18]. As codeine

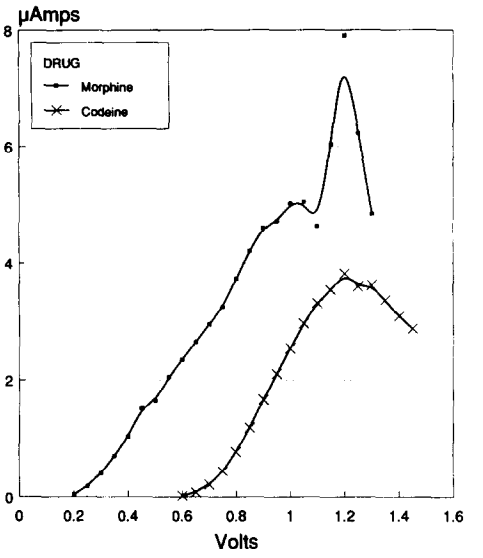

Figure 3
Electrochemical detection of morphine sulphate and codeine phosphate (0.1 g l^{-1}) at pH 2.3. Standards were injected manually (20 µl) using an adapted 7125 Rheodyne injector and chromatographed directly on an HPLC column (Apex II ODS, 15 × 0.45 cm i.d., 5 µm, Jones Chromatography) in a modified mobile phase [acetonitrile–sodium dihydrogen orthophosphate containing sodium dodecylsulphate (5 mM; pH 2.3; 10 mM; 40:60, v/v)]. Voltage on Detector 1 was 0.0 V. Voltage on Detector 2 was initially set at +1.5 V then reduced by 0.5 V steps. Response time for both detectors was 0.4 s.

possesses a methyl substituent blocking the 3-hydroxy group detection at voltages below +0.6 V is not possible. An important factor also, is the rise in background current above +0.8 V. It thus becomes necessary to reduce the detection voltage as far as possible to maintain a satisfactory signal/noise ratio without effectively losing the codeine signal. An optimum of +0.45 V has been used to detect morphine and an active metabolite [1] in a similar study. It was found that an optimum voltage of +0.9 V was best for the measurement of morphine and codeine in the same chromatographic run. Furthermore, when a second detector was set at +0.4 V and a differential mode used (Channel 1–Channel 2), an approximately 3 times improvement of signal/noise resulted. The second detector is being used as a reference electrode in this case [19].

Performance of the assay

The minimum detectable concentrations and correlation coefficients for the three sample matrices, were calculated by a proprietary

Table 2
Experimental parameters for plasma, hydrolysed plasma and stomach contents. All concentrations are given in $\mu g\ l^{-1}$. Values in parentheses refer to concentrations of codeine and morphine in low quality control (LQC), medium quality control (MQC), high quality control (HQC) and running quality control (RQC) spiked samples. Each relative standard deviation (%RSD) refers to an average value of the running quality control samples for that sample matrix. Limit of detection (LOD) is based on a calculated value of the blank plus two standard deviations

	Plasma	Hydrolysed plasma	Stomach contents
Codeine			
LOD	16.976	31.164	10.73
r	0.9977	0.9991	0.9991
LQC (7.5)	8.25	8.61	6.85
MQC (30)	28.53	27.83	26.25
HQC (120)	116.33	114.28	116.81
RQC (40)	34.82	50.21	36.62
%RSD	5.3	4.5	7.5
n	6	6	5
Morphine			
LOD	3.956	9.559	4.627
r	0.9999	0.9993	0.9998
LQC (7.5)	—	—	10.02
MQC (30)	25.73	39.76	24.57
HQC (120)	108.91	119.82	113.68
RQC (40)	38.79	37.52	37.76
%RSD	8.2	8.8	5.0
n	6	6	5

computer program for non-weighted linear regression (Table 2). The limit of detection was based on a calculated value of the blank plus two standard deviations of the blank [20]. Linearity of the calibration line remained excellent over two orders of magnitude. The equations for codeine and morphine, respectively, are as follows. In plasma $y = 508.2290x - 2064.6931$ and $y = 1456.5302x + 1516.9804$; in hydrolysed plasma $y = 120.8884x - 2787.2872$ and $y = 413.5959x + 2327.0958$; in gastric juice $y = 633.8041x - 3079.9310$ and $y = 2724.4511x - 37.7796$. The results of quality control experiments to verify assay linearity and drift were good (Table 2). The pre-column stood up to the required number of repeat determinations on serum, serum plus enzyme and diluted gastric juice (up to 60 analysed) without the need to change the packing material. Sample repeatability had a relative standard deviation (RSD) <9% of an average repeated non-randomized running standard ($40\ \mu g\ l^{-1}$) for all sample types (Table 2). A study of the repeatability of samples and standards over an analytical run was based on one set of duplicates, for a single test experiment. It was found that occasionally, when all results from a run were plotted, single results fell much lower than the accompanying duplicate. It was further noticed that on occasions the autosampler needle became blocked causing irregular dispensing and in some cases a "head motion error" was displayed on the autosampler. The two occurrences appear to be linked. A change from an original 1.0-ml sized sample tube to a smaller volume, cleared up the problem. The recovery of morphine and codeine from aqueous solutions was 99.6 and 96.5%, respectively and from spiked serum was 87.8% (87.7; 87.9) and 86.3% (86.3; 86.3), respectively. The small drop in recovery may be due to protein binding which is surprising considering that methanol had been added to the eluent. It was found that the inclusion of a buffer salt to adjust the pH to 8.5 raised the limit of detection of morphine in gastric juice and hydrolysed serum. A better limit resulted when the wash eluent was replaced with methanol–water (5:95, v/v).

During development the Coulochem 5510A/5011 analytical cell remained uncontaminated for 6 months. This demonstrates the improved removal of protein from the fraction of extracted drug. The automation of the assay has resulted in a precision of <4% RSD for codeine and <2.5% RSD for morphine both from spiked serum. This measured error approaches the reported performance of the manufactured components of the system. As a consequence of this, the precision of the assay

for morphine and codeine over a period of time on real samples is likely to be better than any other conventional method at present available for these drugs.

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