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Determination of codeine in human plasma by high-performance liquid chromatography with fluorescence detection☆

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Abstract

A rapid, reliable and rugged assay for determining codeine in human plasma using reversed-phase high-performance liquid chromatography with fluorescence detection was developed. This analytical method utilized an ion-exchange/mixed-mode solid-phase extraction procedure. The chromatographic separation was achieved using a 150×4.6 mm I.D., 3-μm reversed-phase C_8 (deactivated for basic analytes) column at ambient temperature. Fluorescence detection (excitation at 214 nm and emission above 345 nm) for codeine and nalorphine allowed for a detectable limit of 5 ng/ml. The results showed that the method was linear from 10 to 300 ng/ml. The method had good reproducibility, precision, accuracy and recoveries of 91 and 90% for codeine and nalorphine, respectively. This method has been applied to study the pharmacokinetics of codeine in normal human subjects.

1. Introduction

Codeine has long been used as an analgesic and antitussive in pharmaceutical preparations. A sensitive and specific bioanalytical method is essential for studying the bioavailability of codeine from oral formulations. Many previously reported assays [1–4] for codeine utilize liquid–liquid extractions which are time consuming and solvent-usage intensive. The recoveries associated with these methods [2–4] are often below 80%, and endogenous peaks elute near the codeine or internal standard peaks [5] making quantitation at the lower limits of detection difficult. A high-performance liquid chromatographic (HPLC) method with fluorescence detection has been developed, which is both accurate and precise for the determination of codeine. This method utilizes a solid-phase extraction technique which provides clean extracts with high recoveries for codeine and the internal standard nalorphine. This method may be used in pharmacokinetic studies where monitoring of codeine in human plasma samples is required without metabolites or other compounds interfering. To date, several thousands of samples have been analyzed using this method to de-

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termine the bioavailability of codeine. The solid-phase extraction procedure may readily be automated using any of the commercially available sample processing workstations.

2. Experimental

2.1. Chromatographic conditions

The HPLC system consisted of Hewlett-Packard (Avondale, PA, USA) Series 1050 pump and 1050 autosampler equipped with Applied Biosystems (Ramsey, NJ, USA) Spectroflow 980 programmable fluorescence detector. The fluorescence detector was operated at a 214 nm excitation wavelength with an emission wavelength cutoff filter of 345 nm. The chromatographic separation was performed on a 150 × 4.6 mm I.D., 3-μm Basic C_8 column (YMC, Wilmington, NC, USA). The mobile phase composition consisted of acetonitrile–5 m*M* ammonium phosphate dibasic (8:92, v/v) adjusted to pH 5.8 with phosphoric acid. The flow-rate through the column at ambient temperature was 1.0 ml/min. The column provided excellent resolution of the analytes from all the endogenous peaks present in the extracted plasma. Consistent column performance was found from various lots of packing material. Typically over one thousand injections could be made on a column with no appreciable loss in performance.

2.2. Data handling

Data acquisition and calculations were performed by the Perkin-Elmer Nelson (PE Nelson) Access*Chrom chromatography data analysis system (revision 1.7; Cupertino, CA, USA).

2.3. Sample preparation

Aliquots of 1 ml of plasma were pipetted into 75 × 12 mm I.D. polypropylene test tubes to which 100 μl of the 1000 ng/ml nalorphine internal standard solution and 1.0 ml of deionized water were added. The solid-phase extractions were performed on a Varian Vac-Elut sample processing manifold. The Bond Elut Certify (Varian Sample Preparation Products, Harbor City, CA, USA) columns (3 ml capacity) were conditioned with 2.0 ml of methanol followed by 2.0 ml deionized water. The plasma samples were then transferred to the columns and drawn through at a flow-rate of 2.0 ml/min. The columns were rinsed by using 2.0 ml of water followed by 2.0 ml acetonitrile. The packing was allowed to dry for 1 min under vacuum after the acetonitrile rinse step. The codeine and nalorphine were then eluted into polypropylene test tubes using 2.0 ml of 98% of dichloromethane–isopropanol (80:20, v/v) with 2% ammonium hydroxide. The eluent was then evaporated to dryness under nitrogen at 40°C using a Zymark (Hopkinton, MA, USA) Turbo Vap LV evaporator. The residue was then reconstituted with 100 μl of mobile phase and 60 μl were injected on the HPLC column.

3. Results and discussion

3.1. Linearity and calibration

To evaluate the linearity of the method, standard curves were prepared by spiking plasma with different amounts of codeine in the range of 10 to 300 ng/ml and a constant amount of nalorphine (100 ng/ml). Linear regression analyses were performed using ratios of peak areas of drug to internal standard vs. the respective drug concentrations. To determine the inter- and intra-day accuracy and precision of the calibration curves, three seven-point calibration curves in the range of 10–300 ng/ml were assayed on three separate days. One curve was used to calibrate the other two curves. As shown in Table 1, good inter-/intra-day precision was obtained with the mean accuracy ranging from −8.00 to 2.50% over the investigated concentrations.

The accuracy was estimated by the slope (mean slope value 1.01), intercept (mean intercept value −0.0340) and correlation coefficient (e.g. 0.9999). For example, a typical least

Table 1
Accuracy and precision of codeine calibration curves

Actual concentration (ng/ml)	Calculated codeine concentration (ng/ml)			Mean (ng/ml)	Standard deviation (ng/ml)	Mean accuracy (%)	Relative standard deviation (%)
	Day 1	Day 2	Day 3				
10.0	10.1, 10.5, 11.2	10.1, 10.5, 9.32	8.63, 8.66, 9.89	9.88	0.864	−1.20	8.75
20.0	17.8, 19.2, 19.3	18.9, 18.4, 18.2	19.1, 17.3, 17.8	18.4	0.720	−8.00	3.91
30.0	29.3, 30.8, 30.5	29.8, 31.0, 29.2	29.2, 29.5, 31.0	30.0	0.786	0.00	2.62
50.0	50.9, 50.0, 49.9	48.7, 51.0, 49.2	49.6, 50.3, 49.2	49.9	0.781	−2.00	1.57
100	101, 101, 106	103, 102, 98.9	102, 101, 100	102	2.02	2.00	1.98
200	204, 204, 208	201, 204, 203	207, 210, 205	205	2.76	2.50	1.35
300	297, 306, 302	299, 309, 301	295, 312, 299	302	5.67	0.667	1.88

squares plot gives the equation $y = 0.013x + 0.0151$ with correlation coefficient of 0.9999.

3.2. Precision and accuracy

Inter- and intra-day accuracy and precision were determined by analyzing human plasma controls spiked with codeine at levels of 10.0, 50.0 and 150 ng/ml. The intra-day precision ranged from 1.35% ($n = 4$) to 16.1% ($n = 3$). The inter-day precision ranged from 2.57% ($n = 11$) to 9.75% ($n = 10$).

The accuracy was determined by comparing the measured concentrations to the expected concentrations of codeine in spiked blank human plasma. The mean deviations ranged from −11.8 to −3.20% for 10.0 ng/ml, −3.00 to −0.400% for 50.0 ng/ml and −1.33 to 2.00% for 150 ng/ml codeine in human plasma. Values for precision and accuracy are summarized in Tables 2–4.

Table 2
Precision and accuracy of 10 ng/ml codeine control

Day	Calculated concentration (ng/ml)	Mean (ng/ml)	Standard deviation (ng/ml)	Relative standard deviation (%)	Accuracy (%)
1	8.91				−10.9
	10.0				0.00
	10.2				2.00
	9.59				−4.10
		9.68	0.570	5.89	−3.20
2	9.03				−9.70
	8.21				−17.9
	9.22				−7.80
		8.82	0.537	6.09	−11.8
3	8.68				−13.2
	8.46				−15.4
	11.2				12.2
		9.45	1.52	16.1	−5.50
Overall		9.35	0.912	9.75	−6.50

Table 3
Precision and accuracy of 50 ng/ml codeine control

Day	Calculated concentration (ng/ml)	Mean (ng/ml)	Standard deviation (ng/ml)	Relative standard deviation (%)	Accuracy (%)
1	48.9				−2.20
	49.6				−0.80
	50.8				1.60
		49.8	0.961	1.93	−0.400
2	49.4				−1.20
	49.6				−0.80
	48.3				−3.40
	48.4				−3.20
		48.9	0.670	1.37	−2.20
3	48.9				−2.20
	49.0				−2.00
	45.9				−8.20
	50.1				0.200
		48.5	1.80	3.71	−3.00
Overall		49.0	1.26	2.57	−2.00

Table 4
Precision and accuracy of 150 ng/ml codeine control

Day	Calculated concentration (ng/ml)	Mean (ng/ml)	Standard deviation (ng/ml)	Relative standard deviation (%)	Accuracy (%)
1	144				−4.00
	150				0.00
	152				1.33
	147				−2.00
		148	3.50	2.37	−1.33
2	153				2.00
	151				0.667
	153				2.00
	156				4.00
		153	2.06	1.35	2.00
3	154				2.67
	150				0.00
	143				−4.67
	154				2.67
		150	5.19	3.46	0.00
Overall		151	4.06	2.69	0.667

3.3. Recovery

Absolute recovery was measured by direct comparison of peak areas of non-extracted water standards vs. plasma extracts. The recoveries of codeine and nalorphine, the internal standard, were determined separately. The recovery of codeine over the standard curve concentrations ranged from 82.7 to 108% and averaged 91.4%. The recovery of the internal standard, nalorphine at 100 ng/ml was 90.7%.

3.4. Sensitivity

The working calibration curve for this method is prepared with 10 ng/ml codeine in plasma as the lowest standard. This is considered to be the limit of quantitation of codeine which can be measured within a certain preset level of certainty. The limit of detection was 5 ng/ml, represented as the amount of sample that generates a detector response equal to twice the background noise of the system.

A representative chromatogram of blank plasma containing internal standard (nalorphine) is shown in Fig. 1. There is no chromatographic interference at the retention time of codeine (15 min). The internal standard has a retention time of 18 min. Figs. 2 and 3 are chromatograms of the minimum codeine plasma concentration which can accurately be measured (10 ng/ml) and one of a higher concentration (300 ng/ml).

3.5. Specificity/interferences

Codeine is completely resolved from the internal standard nalorphine, and there are no interferences present at either of the respective retention times. The major metabolites of codeine are morphine and norcodeine. These metabolites were assayed for any possible interferences and the chromatograms are shown in Fig. 4.

The most difficult part in applying a solid-phase extraction technique [6] is identifying a sorbent which will eliminate interfering compounds from the sample matrix. Previous work

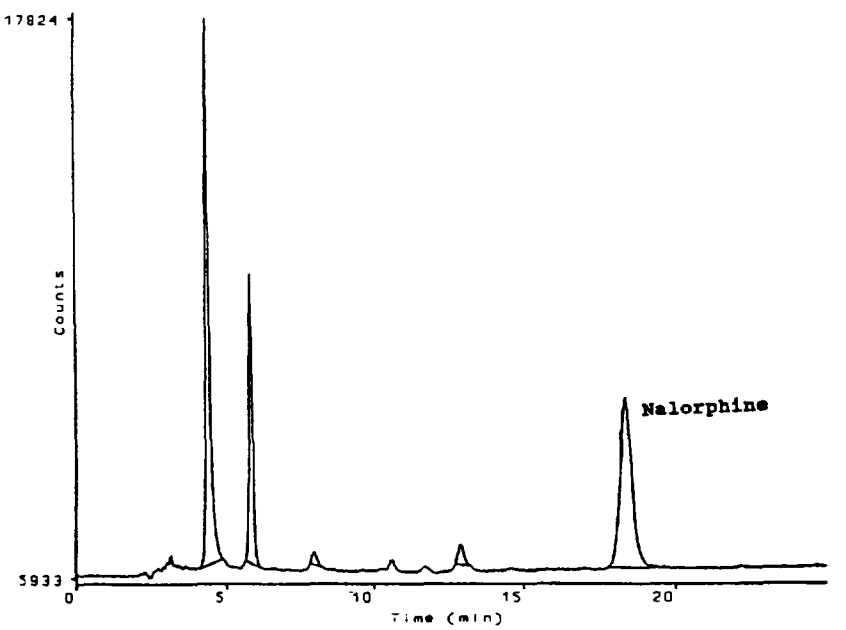

Fig. 1. Chromatogram showing extracted blank plasma spiked with 100 ng/ml nalorphine (internal standard). Chromatographic conditions: injection volume, 60 μl; extraction column, Bond Elut Certify colums (3 ml capacity); analytical column, YMC Basic C_8 (150 × 4.6 mm I.D., 3 μm); mobile phase, acetonitrile–5 m*M* ammonium phosphate dibasic (pH 5.8) (8:92); flow-rate, 1.0 ml/min; fluorometric detection (excitation wavelength 214 nm, emission above 345 nm).

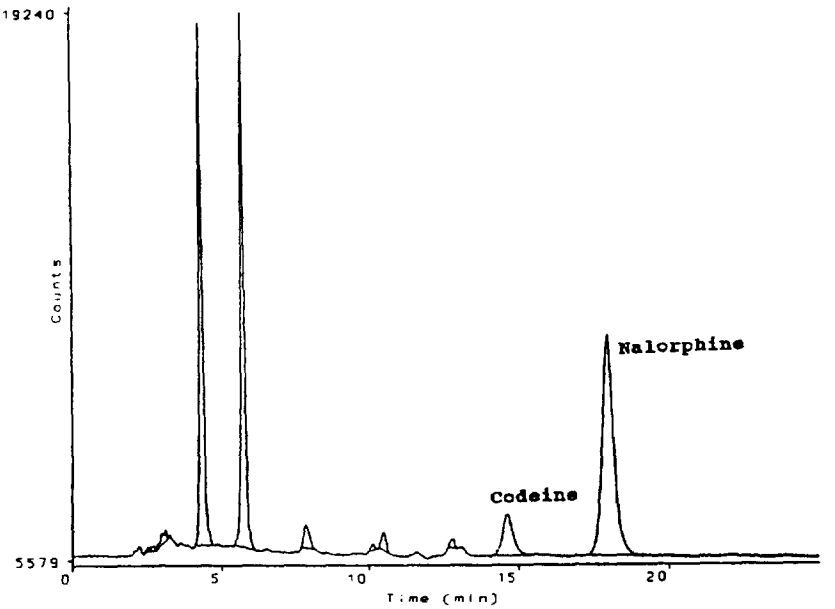

Fig. 2. Chromatogram showing extracted blank plasma spiked with 10 ng/ml of codeine and 100 ng/ml of nalorphine (internal standard). Chromatographic conditions as in Fig. 1.

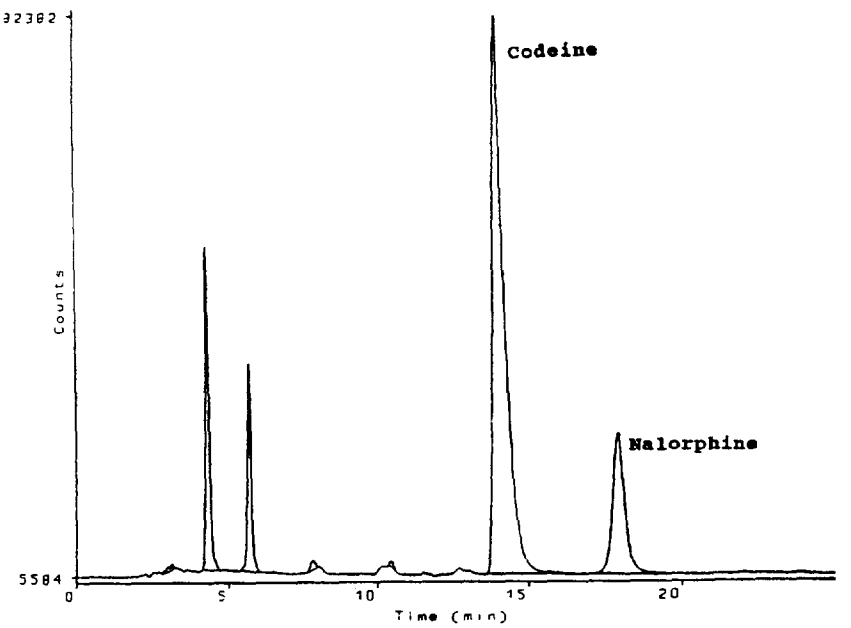

Fig. 3. Chromatogram showing extracted blank plasma spiked with 300 ng/ml of codeine and 100 ng/ml of nalorphine (internal standard). Chromatographic conditions as in Fig. 1.

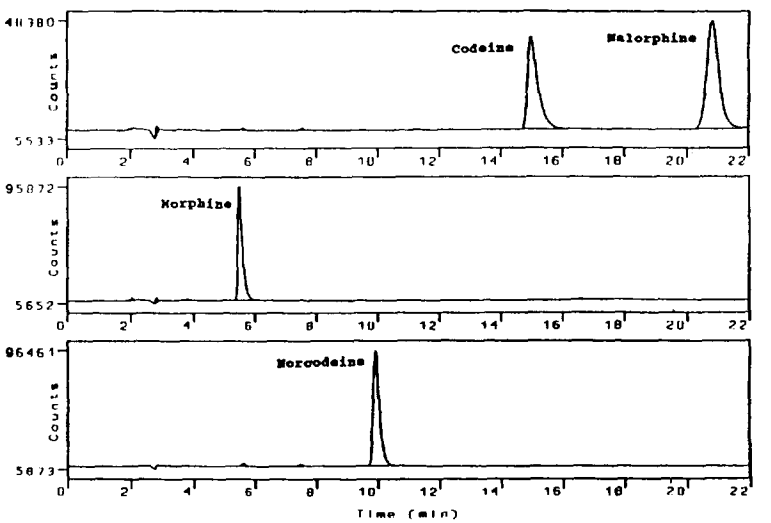

Fig. 4. Water-spiked chromatograms of pure drug compounds: codeine phosphate (500 ng/ml), nalorphine hydrochloride (1000 ng/ml), morphine sulfate (1000 ng/ml) and norcodeine hydrochloride (1000 ng/ml). The top chromatogram is codeine and nalorphine (15 and 22 min, respectively), the middle chromatogram morphine at 5.5 min and the bottom chromatogram norcodeine at 10 min. Chromatographic conditions: injection volume 60 μl; analytical column, YMC Basic C_8 (150 × 4.6 mm I.D., 3 μm); mobile phase, acetonitrile–5 m*M* ammonium phosphate dibasic (pH 5.8) (8:92); flow-rate, 1.0 ml/min; fluorometric detection (excitation wavelength 214 nm, emission above 345 nm).

on codeine was performed on a non-polar C_{18} cartridge, as well as a C_8 cartridge. The advent of the Bond-Elut Certify column (acid/base/neutral sorbent) resulted in a much cleaner plasma extract without almost any spurious peaks in the chromatogram.

3.6. Stability

The stability of codeine in plasma was determined by analyzing frozen plasma standards after one week, two weeks, three months and seven months. These frozen standard curves were compared against a freshly prepared codeine plasma standard curve in each instance. Illustrated in Table 5 are the calculated concentrations (ng/ml) of codeine from the newly prepared and frozen curves. From the results obtained (Table 5), it was concluded that codeine stored frozen in plasma is stable for at least seven months.

3.7. Freeze and thaw

To assess the instability of the analytes due to the number of quantitative freeze thaw cycles, a stock plasma solution of 50 ng/ml concentration of codeine was made and 1-ml aliquots from this stock were pipetted into 10 tubes. Each tube was subjected to different cycles (Table 6) of freezing and thawing. Tube 1 was frozen and thawed once while tube 10 was frozen and thawed ten times. After the cycles were completed the concentration of codeine in these samples were measured against freshly prepared codeine standard curves. The results are illustrated in Table 6.

4. Application

This method has been utilized to assay over 3800 plasma samples from a bioequivalence study in normal volunteers. After obtaining Institutional Review Board approval and Informed Consent, subjects received two formulations of codeine (100 mg) both administered under fed and fasted conditions in a four-way randomized crossover study. As can be seen from Figs. 5 and 6, the 10 and 150 ng/ml quality

Table 5
Stability of codeine in plasma

Actual concentration (ng/ml)	calculated codeine concentration (ng/ml)							
	One week		Two weeks		Three months		Seven months	
	New curve	Frozen curve	New curve	Frozen curve	New curve	Frozen curve	New curve	Frozen curve
10.0	8.87	9.35	9.19	9.55	9.44	11.4	11.6	9.82
20.0	19.3	20.2	19.9	19.5	19.5	18.4	22.3	19.4
30.0	28.8	29.8	28.7	29.7	32.0	28.8	27.5	27.0
50.0	51.9	51.3	50.9	50.0	50.0	47.5	44.1	45.2
100	103	99.1	104	102	99.3	92.8	102	94.0
200	198	198	197	203	198	188	207	190
300	300	308	301	308	301	291	296	287
Linear regression equations	$y = 0.013x + 0.0071$ $r = 0.9999$	$y = 0.013x - 0.0081$ $r = 0.9995$	$y = 0.013x + 0.0153$ $r = 0.9996$	$y = 0.013x + 0.0002$ $r = 1.000$	$y = 0.014x + 0.0195$ $r = 0.9999$	$y = 0.014x + 0.0085$ $r = 0.9994$	$y = 0.013x + 0.0361$ $r = 0.9992$	$y = 0.013x + 0.0207$ $r = 0.9999$

r = Correlation coefficient.

Table 6
Freeze and thaw

Day	Codeine concentration (ng/ml)
1	46.9
2	46.2
3	50.2
4	49.4
5	50.3
6	50.8
7	48.6
8	49.4
9	48.9
10	49.4

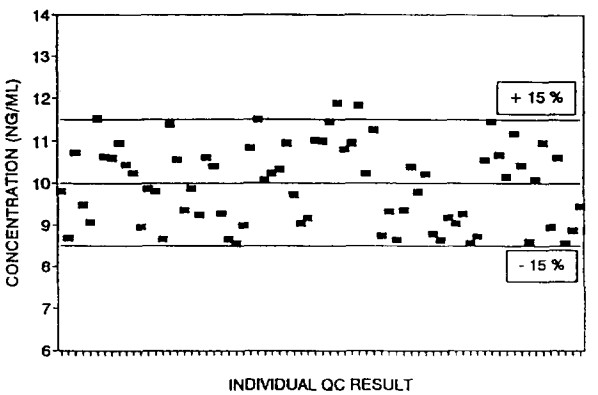

Fig. 5. Results from quality control (QC) samples (concentration 10 ng/ml) during the time course of the pharmacokinetic study.

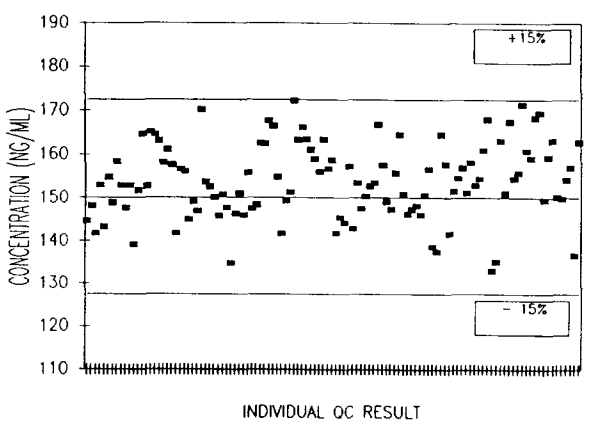

Fig. 6. Results from quality control samples (concentration 150 ng/ml) during the time course of the pharmacokinetic study.

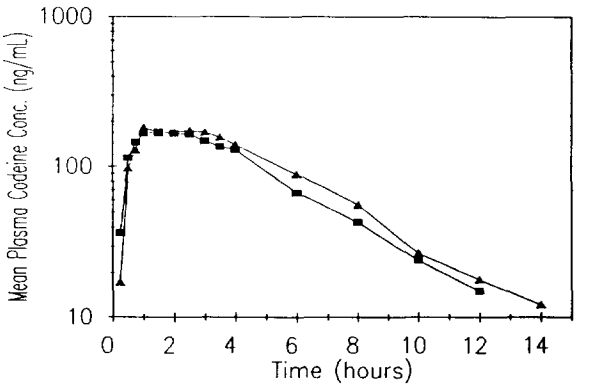

Fig. 7. Semilogarithmic plot of mean plasma concentration versus time profile for codeine after a single dose of 100 mg codeine phosphate liquid fed (▲) and fasted (■) in human volunteers.

control data demonstrate the good overall inter/intra-day accuracy and precision of the method under routine conditions obtained during the 3 month analysis period. Fig. 7 shows mean plasma concentrations of codeine following a single 100-mg dose of codeine phosphate liquid with and without food. The terminal half-life of codeine was 2.5 h.

5. Conclusions

The analysis of codeine in human plasma by HPLC with fluorescence detection is a rapid, sensitive and specific assay. This analytical method is capable of quantitating and monitoring the levels of codeine in plasma following an ion-exchange/mixed-mode solid-phase extraction procedure.

The use of the YMC Basic C_8 column resulted in excellent separation of codeine and nalorphine (internal standard) as well as good peak shape. There were no interferences with the analytes from extracted endogenous fluorogenic substances.

Combining both the extraction technique and detection method has resulted in a method suitable for determining the plasma codeine concentrations in pharmacokinetic studies.

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