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The study of codeine–gluthetimide pharmacokinetic interaction in rats

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Abstract

A high-performance liquid chromatographic (HPLC) assay with native fluorescence detection was developed for the simultaneous quantification of codeine and its two metabolites, morphine and morphine-3-glucuronide (M-3-G), in rat plasma. Solid-phase extraction was used to separate codeine and its metabolites from plasma constituents. Extraction efficiencies of codeine, morphine and M-3-G from rat plasma samples were 97, 92 and 93%, respectively. The chromatographic separation was performed using a reversed-phase C18 column and an elution gradient at ambient temperature. Using native fluorescence detection (excitation at 245 nm and emission at 345 nm), the detection limits of 50 ng/ml for morphine, 25 ng/ml for codeine and 20 ng/ml for M-3-G were obtained. The method had good precision, accuracy and linearity, and was applied to the study of glutethimide's influence on codeine metabolism in rat, following single doses of codeine–glutethimide association. The results confirmed the fact that glutethimide was responsible for a significant increase of morphine plasma levels and for their maintenance in time, concomitant with a significant decrease of M-3-G plasma levels, explained by the inhibition of morphine glucuronidation. In conclusion, glutethimide potentiates and prolongs the analgesic effect of codeine by a pharmacokinetic mechanism.

Keywords: HPLC; SPE; Codeine metabolism; Codeine–glutethimide interaction

1. Introduction

Codeine (Cd), an opioid alkaloid, is used in therapy for its antitussive, analgesic and antidiarrheic effects. Glutethimide (Glth), a piperidinedione derivative, is a hypnotic-sedative drug with pharmacological activity similar to barbiturates [1]. In 1970s, the first cases of codeine–glutethimide combination abuse were observed [2,3]. Administered concomitantly, these two drugs can induce an euphoric state, similar but longer than that produced by heroin, the association being considered a substitute of this drug of abuse. As the substances are easily obtained in different pharmaceutical preparations and are taken orally, the number of abusers using this or similar combinations is lately constantly growing especially in the Eastern Europe [4–8].

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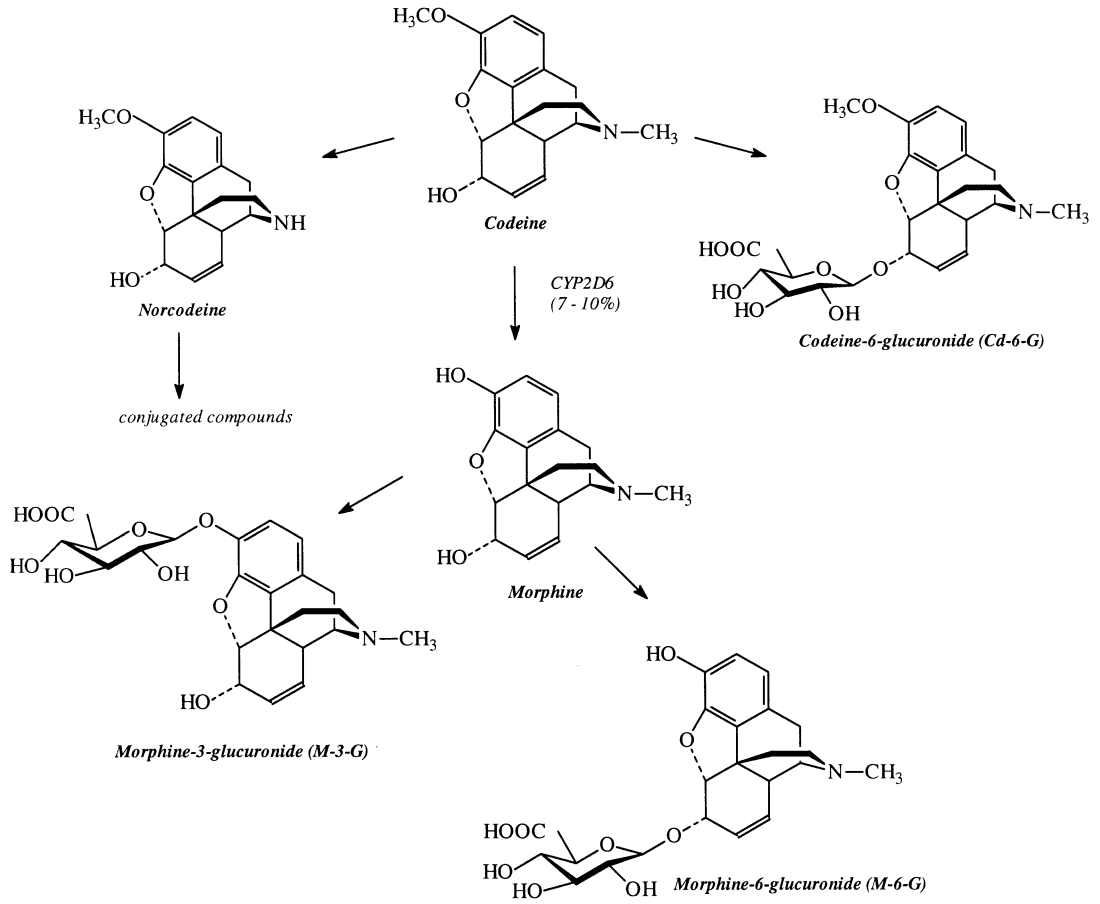

Fig. 1. The main biotransformation pathways of codeine.

The mechanism of codeine–glutethimide interaction is not yet explained, but it is supposed that it involves both a synergistic pharmacological interaction, as both drugs are CNS-depressants, and a pharmacokinetic interaction, as glutethimide is an enzyme-inducer [1].

One of the objectives of our research was to develop a sensitive, selective and precise analytical method that can be used to study the influence of glutethimide on codeine metabolism in Wistar rats.

Codeine pharmacological activity is due to its biotransformation to morphine (Fig. 1). This reaction is catalyzed by the isoenzyme CYP2D6, corresponding to CYP2D1 in rats, which is subjected to a genetic polymorphism, known as the debrisoquine/sparteine polymorphism [9,10]. Morphine is metabolized extensively by at least two isoenzymes of uridine-diphosphate-glucuronosyltransferases (UDPGT) [11,12]. Morphine glucuronides are pharmacologically active: morphine-6-glucuronide (M-6-G) has analgesic activity superior to morphine [11,13,14], while morphine-3-glucuronide (M-3-G) has an anti-analgesic activity competitive to morphine and M-6-G and stimulates the CNS [11,15].

The conjugated metabolites can be analyzed directly only by HPLC and there have been published a number of papers on the simultaneous quantification of opioids and their metabolites in biological fluids using HPLC and solid phase extraction (SPE) [15–21].

The UV detector is not sensitive enough to detect opioids and their metabolites in blood, as

Table 1
Therapeutic, toxic and lethal concentrations of morphine and codeine in human plasma

Substance	Therapeutic concentrations (mg/l)	Toxic concentrations (mg/l)	Lethal concentrations (mg/l)
Morphine	<0.10	0.10–0.50	0.10–4.00
Codeine	0.01–0.25	0.20–1.00	1.80

both therapeutic and toxic plasma levels of morphine and codeine are quite low (Table 1) [22,23]. The fluorescence detector is more sensitive and selective compared with the UV detector, the signal is stable and reproducible and it is preferred for the quantitative analysis. The opioids have native fluorescence and a number of articles have been published on the use of HPLC with the detection of native fluorescence for their quantification in blood samples [16,24–27].

Considering the previously reported research and the fact that M-6-G is not formed in rats [28], the objective of our study was the isolation of codeine and two of its metabolites: morphine and M-3-G by solid phase extraction, followed by HPLC separation and quantification by the detection of their native fluorescence. The method was validated and applied to the study of codeine–glutethimide interaction in Wistar rats in single dose administration.

2. Experimental

2.1. Standards and reagents

Morphine hydrochloride (M·HCl), codeine phosphate (Cd·PO_4) and glutethimide (Glth) of pharmaceutical grade were obtained from TERAPIA S.A. (Cluj-Napoca, Romania). Morphine-3-beta-glucuronide (M-3-G) of HPLC grade was obtained from the Scientific Section, PDAB/DOA/UNCDP (Vienna, Austria).

The stock solutions of morphine (1 mg/ml), codeine (1 mg/ml) and M-3-G (28 μg/ml) were prepared in Millipore water and were stored at 4 °C. The control solutions used to validate the HPLC method were prepared by diluting the stock solutions in the mobile phase (solvent B).

All the solvents and reagents were of analytical or HPLC grade. Natrium fluoride, acetonitrile and methanol were from Merck (Darmstadt, Germany), the formic acid (80% solution) and ammonia from Chimopat S.A. (Bucharest, Romania), triethylamine from Carlo Erba (Milano, Italy) and ammonium sulfate from Reactivul S.A. (Bucharest, Romania). The pure Millipore water was freshly prepared.

The ammonium sulfate 500 mM (pH 9.3) was prepared by dissolution (6.607 g/l) in Millipore water and adjustment at pH 9.3 with ammonia. This solution was then diluted 1:100 in Millipore water and the pH was adjusted with ammonia at 9.3 to obtain the ammonium sulfate 5 mM (pH 9.3).

2.2. Experimental protocol

Male Wistar rats (from the Animal Breeding Station of the University of Medicine and Pharmacy from Cluj-Napoca) that were maintained in standard conditions were used in the experiment.

The study of codeine–glutethimide interaction was performed on two groups of 33 rats each (140 ± 8 g). The substances were administered by oral intubation in a single dose as follows: group 1 received 1/10 of the median lethal dose (LD_{50}) of Cd·PO_4 (42.7 mg/kg body weight) associated with 1/10 of LD_{50} of Glth (60 mg/kg body weight) from a suspension in distilled water prepared with Tween 80; group 2 received 1/10 of LD_{50} of Cd·PO_4 (42.7 mg/kg body weight) from a solution in distilled water.

At 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 720 min after the intubation, three rats from each group were killed by decapitation and the blood was collected on natrium fluoride. The plasma separated after centrifugation (10 min, $3500 \times g$) was stored at -20 °C till the analysis. After

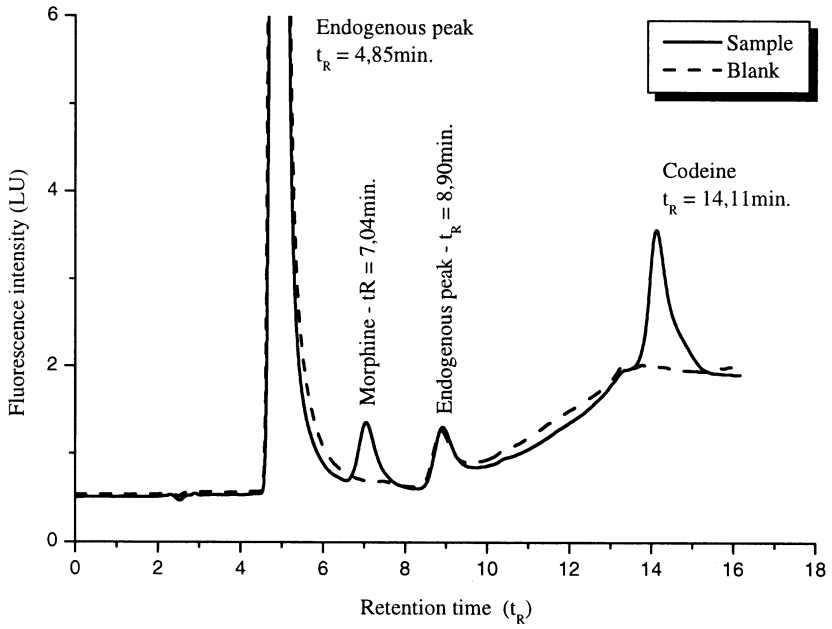

Fig. 2. The chromatogram of a plasma sample spiked with morphine and codeine (600 ng/ml plasma) and of a blank plasma.

defreezing, the plasma was again centrifuged and was extracted on solid phase.

In the process of optimization and validation of the method, plasma samples from non-treated rats were used, spiked with different quantities of morphine, codeine and M-3-G.

2.3. Solid phase extraction

The cartridges (Snap-Cap C18, 300 mg, ABL&E Jasco Cluj-Napoca, Romania) were conditioned with 5 ml methanol, 5 ml Millipore water and 5 ml ammonium sulfate 500 mM (pH 9.3). The plasma sample, diluted at 3 ml with ammonium sulfate 500 mM (pH 9.3) was applied on the columns, which were afterwards rinsed with 5 ml ammonium sulfate 5 mM (pH 9.3). The analytes retained were eluted with 2 ml methanol containing 0.5% triethylamine. The eluent was evaporated to dryness at 40 °C using a Heidolph 94200 (Bioblock Scientific) evaporator. The residue was reconstituted with 500 μl mobile phase (solvent B) and 50 μl were injected into the HPLC column.

2.4. Chromatographic conditions

The HPLC system (HP 1100) consisted of two pumps, an autosampler and a fluorescence detector. The chromatographic separation was performed on a C18 Spherisorb column (250 × 4 mm, 5 μm) with a C18 guard column (30 × 4.6 mm). The flow-rate was 1 ml/min and analyses were performed at ambient temperature. The mobile phase consisted of solvent A—40% (v/v) acetonitrile in solvent B, and solvent B—0.5% (v/v) triethylamine and 0.15% (v/v) formic acid in pure water (pH adjusted to 6.2 with formic acid). The best separations of the analytes from rat plasma were obtained with the elution gradient: 0–5 min ~90% B, 5–10 min ~from 90 to 60% B; 10–14 min ~60% B; 14–15 min ~from 60 to 90% B. The detection was based on the native fluorescence, with excitation at 245 nm and emission at 345 nm.

2.5. Validation of HPLC method

The SPE-HPLC method was studied for the following plasma concentration range: 150–1200

Table 2
Accuracy and precision of the method for the standard solutions used for calibration (n = 5)

	Theoretic concentration (ng/ml)[a]	Concentration found (ng/ml)[a]	S.D.	R.S.D. (%)	Mean accuracy (A%)	Linearity parameters
Morphine hydrochloride	25	27.50	1.27	4.61	+10	A = 0.2637
	50	48.10	0.18	0.38	−3.80	B = 0.0306
	100	103.33	2.03	1.96	+3.33	r = 0.9999
	300	298.43	4.59	1.53	−0.52	
	600	593.11	4.13	0.69	−1.14	
	900	902.42	9.29	1.03	+0.26	
	1200	1200.74	5.87	0.48	+0.06	
Codeine phosphate	25	22.85	0.49	2.17	−8.60	A = 0.2626
	50	51.35	0.99	1.93	+2.70	B = 0.0605
	100	98.63	2.35	2.38	−1.37	r = 0.9998
	300	298.39	2.69	0.90	−0.57	
	600	605.16	7.03	1.16	+0.86	
	900	906.41	8.25	0.91	+0.71	
	1200	1199.12	4.42	0.36	−0.07	
M-3-G	80	85.13	2.32	2.79	+3.93	A = −0.2966
	200	193.35	2.90	1.50	−3.32	B = 0.0273
	400	405.43	8.97	2.21	+1.35	r = 0.9999
	800	798.73	12.84	1.60	−0.15	
	1600	1600.42	26.13	1.63	+0.02	

[a] Concentrations are expressed in ng alkaloid-base/ml).

ng morphine/ml, 100–1200 ng codeine/ml and 200–1600 ng M-3-G/ml.

The linearity was studied based on three calibration curves on the first validation day, and two other calibrations in 2 other days. The results were evaluated by calculation of a regression line by the least squares method.

The precision and the accuracy were studied in 3 different days (n = 6, k = 3) for five control solutions of different concentrations for each analyte,

Table 3
Accuracy and precision for the plasma samples spiked with different concentrations of morphine, codeine and M-3-G, used for calibration (n = 5)

	Theoretic concentration (ng/ml)	Concentration (ng/ml)	Found (%)	S.D.	R.S.D. (%)	Linearity parameters
Morphine hydrochloride	150	132.34	88.14	9.40	14.13	A = −0.2018
	300	275.22	91.74	27.98	10.17	B = 0.0325
	600	530.13	88.35	42.67	8.05	r = 0.9925
	900	927.53	103.06	86.16	9.29	
	1200	1082.97	90.25	22.95	2.12	
Codeine phosphate	100	90.87	90.87	8.72	9.60	A = −0.7582
	150	133.17	88.78	5.68	4.27	B = 0.0630
	300	297.41	99.14	10.85	3.65	r = 0.9999
	600	613.67	102.28	18.96	3.09	
	1200	1232.14	102.68	5.29	0.43	
M-3-G	200	183.77	91.88	6.28	3.42	A = −0.2739
	400	355.62	88.90	20.73	5.83	B = 0.0256
	800	790.90	98.86	47.21	5.97	r = 0.9990
	1200	1121.25	93.44	86.89	7.75	
	1600	1489.25	93.08	51.82	3.48	

Table 4
Precision and accuracy of the method for standard solutions of different concentrations of morphine hydrochloride, codeine phosphate and M-3-G

	Theoretic concentration (ng/ml)[a]	R.S.D. (%) intraday (n = 6)	R.S.D. (%) interday (n = 18)	Mean accuracy (A%) (n = 18)
Morphine hydrochloride	25	4.79	5.01	+10.00
	300	2.57	2.57	−0.52
	600	2.64	3.04	−1.14
	900	3.82	3.63	+0.26
	1200	4.08	3.63	+0.06
Codeine phosphate	25	6.56	7.27	−8.60
	300	2.00	2.27	−0.57
	600	3.61	3.76	+0.86
	900	1.33	1.44	+0.71
	1200	1.80	0.83	−0.07
M-3-G	80	4.09	5.07	+3.93
	200	0.94	1.92	−3.32
	400	2.20	2.21	+1.35
	800	2.52	2.58	−0.15
	1600	2.47	2.65	+0.02

[a] Concentrations expressed in alkaloid-base.

and for three spiked plasma samples of different concentrations, respectively. The intraday (n = 6) and interday (n = 18) coefficients of variation (R.S.D.%) and the mean accuracy (A%) for all the determinations (n = 18) were calculated.

The limits of detection were appreciated as the quantity of substance that induced a detector response equal to twice the height of the noise. The lower limits of quantification (LLOQ) were calculated based on the standard deviation of the response and the slope: $LLOQ = 10\sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.6. Statistic analysis

The results were analyzed statistically by the analysis of variance and post-tests for a 95% level of confidence.

Table 5
Precision and accuracy of the method for plasma samples spiked with different concentrations of morphine, codeine and M-3-G

	Theoretic concentration (ng/ml)[a]	Concentration found (%)	R.S.D. (%) intraday (n = 6)	R.S.D. (%) interday (n = 18)
Morphine hydrochloride	300	91.74	10.21	12.84
	600	88.35	7.22	10.35
	1200	90.25	8.13	4.85
Codeine phosphate	300	99.14	4.33	3.91
	600	102.28	2.40	3.32
	1200	102.68	2.17	1.52
M-3-G	400	88.90	6.50	8.42
	800	98.86	8.79	5.45
	1600	93.08	4.20	3.32

[a] Concentrations expressed in alkaloid-base.

3. Results

3.1. Validation of HPLC method

Morphine, codeine and M-3-G were HPLC separated at retention times of 7.0, 14.1 and 3.93 min, respectively. The method selectivity is ensured by the selectivity of the fluorescence detector and by the HPLC separation conditions (Fig. 2).

The results show a good linearity ($r > 0.9998$ for standard solutions, and $r > 0.9925$ for spiked plasma samples, respectively), the calibration being precise (R.S.D.% < 11%) and accurate (the percentage of recovery is in the accepted limits: 85–115%) for all the studied concentrations (Tables 2 and 3).

The method was precise and accurate, the values for R.S.D.% and A% being within 15% of the actual value for all the determinations (Tables 4 and 5). For the plasma analysis, the values R.S.D.% and the percentages of recovery were in the accepted limits for concentrations of analytes in biological fluids in the range of micrograms [29], which proved the efficiency of the SPE-HPLC elaborated method.

The limits of detection were for plasma assays: 50 ng/ml for morphine, 25 ng/ml for codeine and 20 ng/ml for M-3-G. The limits of quantification calculated theoretically were: 150 ng/ml for morphine, 100 ng/ml for codeine and 80 ng/ml for M-3-G.

The SPE-HPLC method studied was precise, accurate and linear and was applied in codeine–glutethimide interaction study.

3.2. Codeine–glutethimide interaction in rats

Glutethimide influenced codeine metabolism in rats, increasing significantly the plasma levels of morphine, the main metabolite, and maintaining them at an increased level for a longer period. Concomitantly, a significant decrease of M-3-G (morphine's metabolite) plasma level was observed (Fig. 3). Glutethimide diminishes approximately three times codeine's O-demethylation to morphine and diminishes ~14 times morphine's glucuronidation to morphine-3-glucuronide (Table 6). Consequently, the ratio of plasma molar concentrations: [M-3-G]/[M], which reflect the degree of analgesia induced [30], was much smaller in rats treated with codeine–glutethimide association than in rats treated only with codeine ($P <$

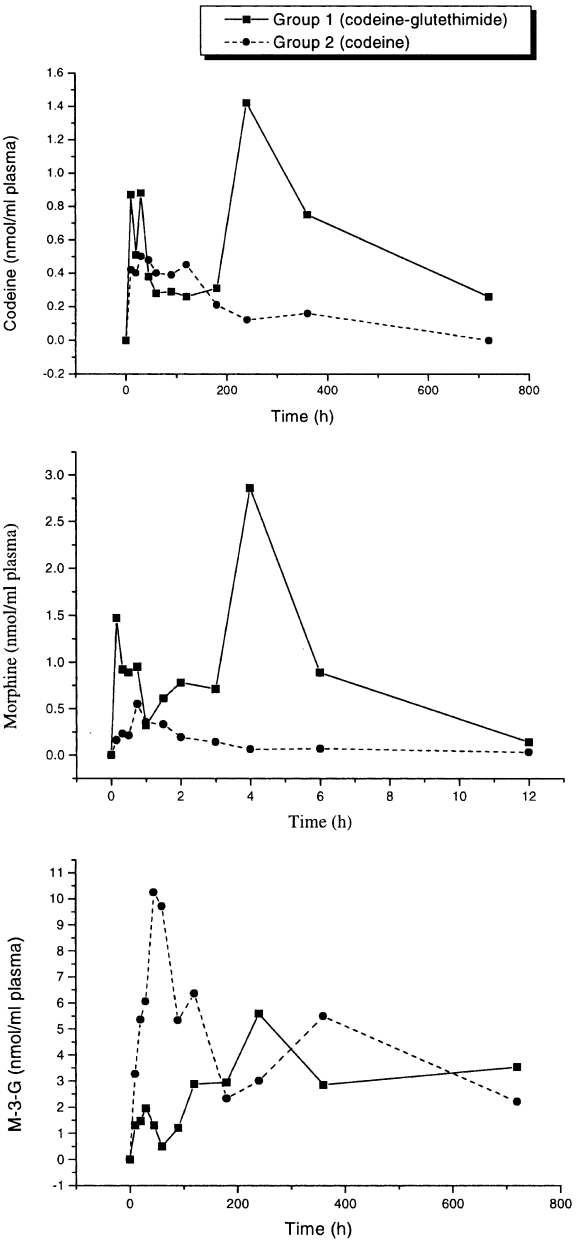

Fig. 3. The influence of glutethimide on codeine, morphine and M-3-G plasma levels in rats treated with codeine–glutethimide (group 1) and codeine (group 2), respectively.

Table 6
The plasma molar concentration ratio (RM) for the two treated groups presented comparatively (*$P < 0.005$)

	Group 1 (codeine–glutethimide)	Group 2 (codeine)
$RM_{codeine's\ O\text{-}demethylation} = [Cd]/([M]+[M\text{-}3\text{-}G])$	0.19 ± 0.09*	0.06 ± 0.03
$RM_{morphine's\ glucuronidation} = [M]/[M\text{-}3\text{-}G]$	0.49 ± 0.29*	0.035 ± 0.02
Evaluation of the analgesia degree: [M-3-G]/[M]	2.28 ± 1.07*	23.14 ± 6.31

5×10^{-8}; Fig. 4). The maximum analgesic effect was obtained 10 min after the administration of the association ([M-3-G]/[morphine] = 0.9) and was maintained for approximately 1 h ([M-3-G]/[morphine] $\leq$ 1.5). It was shown that glutethimide potentiates the analgesic effect of codeine and interferes in its maintenance in time.

4. Discussion

Morphine glucuronides have different polarity compared with morphine and codeine, and elute quickly having small values for the capacity factors (**k**). To obtain acceptable **k** values for the tested analytes, we had to use an elution gradient. The mobile phase that was used ensured a quick reequilibration of the column after each gradient (2 min). The role of triethylamine in the mobile phase was to block the free silanol groups of the stationary phase and to improve the shape of the peaks. The formic acid (80%) has the role to improve the partition of the analytes due to its saline effect, and respectively, to maintain the pH of the mobile phase.

The sensitivity of the detector's response was investigated at three excitation wavelengths (λ_{ex}): 245, 214 and 285 nm (wavelength corresponding to the maximum of absorbance for the three analytes). The height of the noise (h_N) and the height

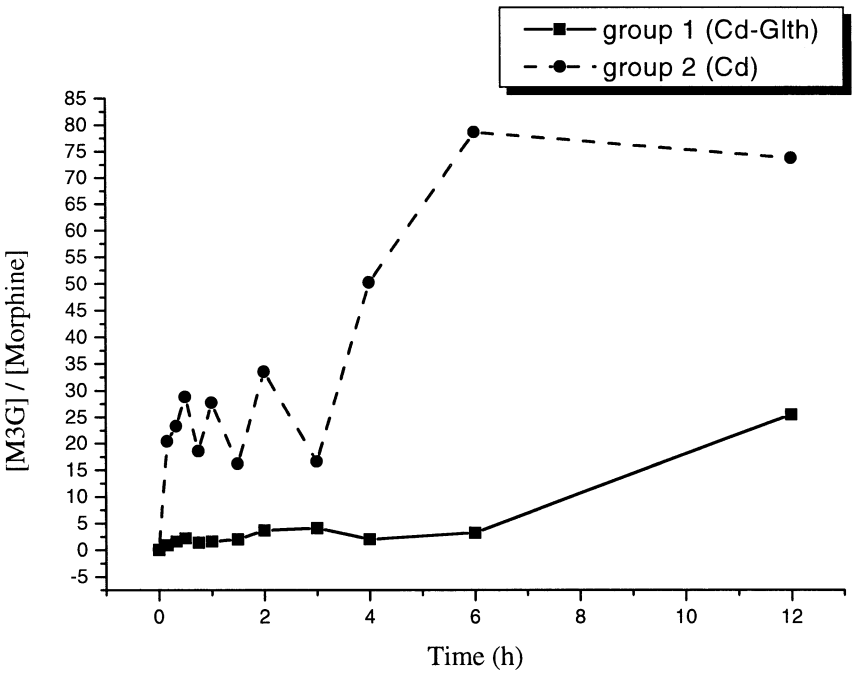

Fig. 4. The dynamic of the plasma molar concentration ratio [M-3-G]/[morphine] in time, an indicative of the degree of analgesia, presented comparatively for the two groups, treated with codeine–glutethimide (group 1), and with codeine (group 2), respectively.

Table 7
Sensitivity of fluorescence detector response according to the excitation wavelength

Substance	$\lambda_{ex} = 245$ nm ($h_N = 0.005$)		$\lambda_{ex} = 214$ nm ($h_N = 0.025$)		$\lambda_{ex} = 285$ nm ($h_N = 0.002$)	
	h_a	h_a/h_N	h_a	h_a/h_N	h_a	h_a/h_N
M-3-G	0.240	43	0.710	28	0.180	90
Morphine	0.140	25	0.300	12	0.095	48
Codeine	0.160	29	0.270	10	0.094	47

h_a, height of analyte peak; h_N, height of the noise.

of analytes' peaks (h_a) for the same sample and the same emission wavelength ($\lambda_{em} = 345$ nm) were compared (Table 7). The best results were obtained with excitation at 245 nm because, even if at 285 nm the ratio h_a/h_N had the highest values, the signal of the analytes at this wavelength was less intense.

The SPE-HPLC method studied for the assay of morphine, codeine and M-3-G in rat plasma samples was precise, accurate, linear (Tables 4

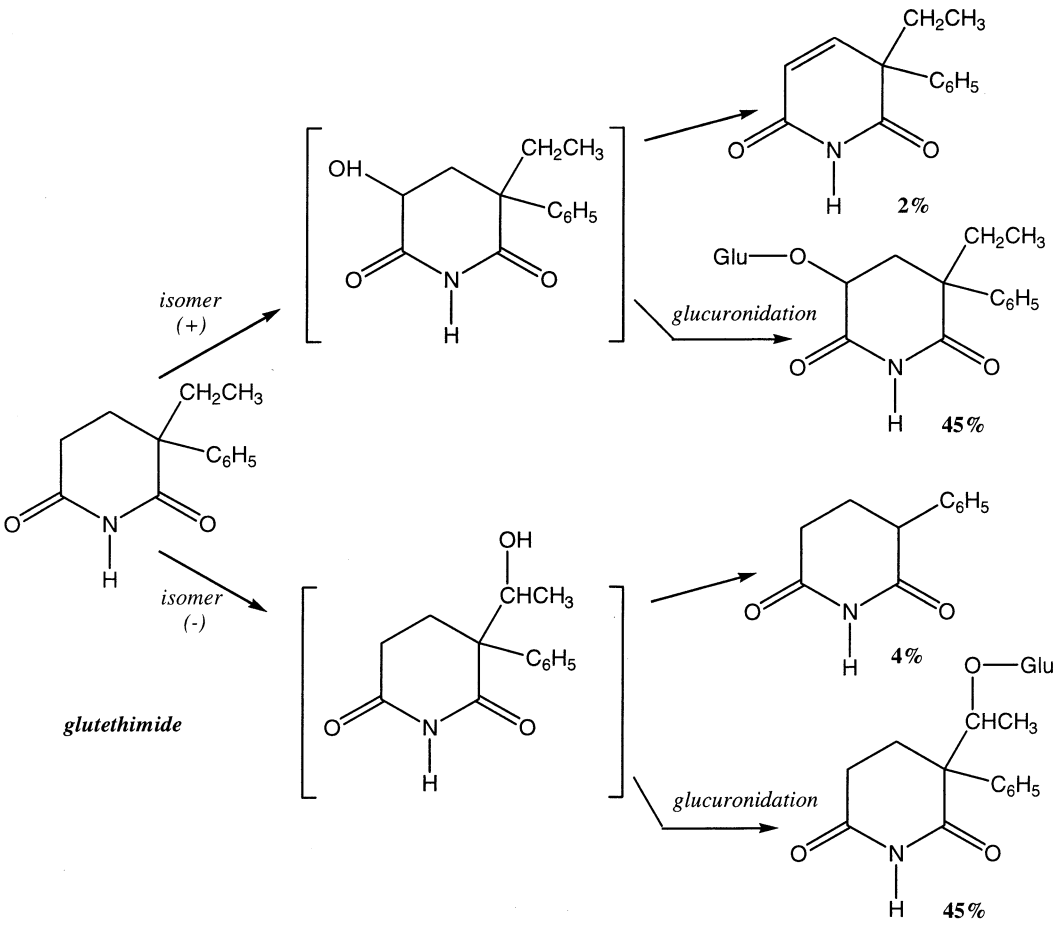

Fig. 5. The main biotransformation pathways of glutethimide.

and 5) and enough sensitive to be applied in studies of pharmacokinetics, metabolism or drug interactions of codeine.

Glutethimide modified codeine's metabolism in rats, following acute administration. The increase of codeine's plasma levels when associated with glutethimide (Fig. 3) could be explained by the decrease of O-demethylation rate. CYP2D6, responsible for the O-demethylation of codeine to morphine, catalyses mainly hydroxylation and dealkylation reactions [31], being involved in the hydroxylation of enatiomers due to its substrate stereoselectivity [9]. Glutethimide is metabolized mainly by hydroxylation, the two optical isomers having different metabolic pathways (Fig. 5). The enzyme systems involved in the metabolism of glutethimide are not yet known. There is a great probability that CYP2D6 should be responsible of at least two of the hydroxylation reactions, glutethimide partially blocking the isoenzyme, which would explain the increase of codeine plasma concentrations and the prolongation of the elimination.

The increase of morphine plasma levels, concomitantly with maintenance of low M-3-G levels after the administration of the association (Fig. 3), as well as the 14 times increase of the metabolic ratio of morphine glucuronidation (Table 6) are suggesting that the hydroxylated metabolites of glutethimide are conjugated by the same UDPGT isoenzymes as morphine, the glucuronidation of morphine being thus blocked by competitive inhibition. Consequently, the metabolic clearance of morphine diminished and morphine plasma levels increased, the elimination rate of morphine being decreased.

The significant decrease of plasma molar concentration ratio, [M-3-G]/[morphine], by glutethimide indicates that the administration of codeine–glutethimide association induces a more pronounced analgesic effect and which is maintained for a longer period compared with codeine single administration. These results are in agreement with those obtained previously in our laboratory in the study of codeine–glutethimide pharmacodynamic interaction in mice (the method of thermic stimulus) [32].

In consequence, the interaction of codeine and glutethimide in acute administration can be explained both by a pharmacodynamic and a pharmacokinetic mechanism.

5. Conclusions

A SPE-HPLC method for the quantification of morphine, codeine and M-3-G in rat plasma samples was elaborated. The method was proved to be very efficient and was applied to the study of codeine–glutethimide drug interaction.

After acute administration in rats, glutethimide inhibited the glucuronidation of morphine, codeine's main metabolite, which was clinically revealed by the potentiation of codeine's analgesic effect.

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