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DETERMINATION OF NANOGRAM AMOUNTS OF AROMATIC COMPOUNDS BY SPECTROPHOTOMETRY ON THIN-LAYER CHROMATOGRAMS

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SUMMARY

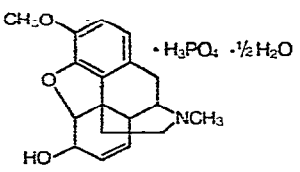
A highly sensitive method is described for the simultaneous determination of codeine and chlorpheniramine in plasma. Thin-layer chromatography is used for the separation of the drugs. The spots are then rendered visible by nitration of the substances on the thin-layer plate. Codeine can be quantified by direct measurement of the resulting fluorescence. After reduction, the aromatic amines are diazotized and coupled with N-(1-naphthyl)ethylenediamine on the thin-layer plate. For codeine the fluorimetric measurement is more reliable than the colorimetric determination. The sensitivity limits are 8 ng/ml of plasma for codeine phosphate and 1–2 ng/ml of plasma for chlorpheniramine maleate. This procedure is also applicable to other aromatic compounds which can be nitrated by the described method. The method has been applied to compare a determination of the plasma levels of codeine and chlorpheniramine after administration in normal capsules and retard tablets.

INTRODUCTION

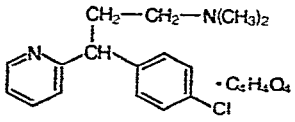
Codeine phosphate in combination with chlorpheniramine maleate is a widely used antitussive. There are many analytical methods for the quantitation of these drugs; however, these methods either lack the required specificity and sensitivity or are laborious and lengthy. This paper describes a sensitive and specific thin-layer chromatographic (TLC) method for the determination of codeine and chlorpheniramine in human plasma. The procedure is also applicable to other aromatic compounds which can be nitrated on thin-layer plates. The main objective of the project was to compare the plasma levels of codeine and chlorpheniramine after oral administration of the drugs either in normal capsules or in a retard formulation.

After oral administration of 15 mg of codeine, Schmerzler *et al.*[1] found maximum serum levels of less than 50 ng/ml. In the metabolic study of Peets *et al.*[2], who administered 12 mg of [^{3}H]chlorpheniramine maleate orally, the average plasma levels of the unchanged drug were *ca.* 17 ng/ml. According to my findings, the values mentioned in the papers of Hanna and Tang[3] and Fischer *et al.*[4] are considered too high. Since I wanted to determine both drugs in the same plasma extract, it was necessary

to develop a method with a sensitivity of 10 ng/ml of plasma for codeine phosphate and of 2 ng/ml of plasma for chlorpheniramine maleate. Both drugs contain a benzene ring (Fig. 1), so that the methods of Kern[5] or Albert and Connors[6] could be applied, which determine aromatic compounds at the microgram level. One of these principles could be adapted to TLC, and reached the sensitivity required for the determination of both compounds at the therapeutic plasma levels.



Codeine phosphate



Chlorpheniramine maleate

Fig. 1. Structure formulas.

PRINCIPLE OF METHOD

After alkalinization of the plasma samples, codeine and chlorpheniramine are extracted with diethyl ether. Following back-extraction into hydrochloric acid and alkalinization, the drugs are re-extracted with dichloromethane. The organic phase is concentrated and applied to a thin-layer silica gel plate. After separation by chromatography, the aromatic substances are nitrated on the plate. Following this reaction codeine shows an intensive fluorescence, which allows the direct quantitation with a suitable scanner. The aromatic nitro-compounds are then reduced with titanium(III) chloride to the amines, which can be diazotized and coupled with N-(1-naphthyl)-ethylenediamine. Codeine and chlorpheniramine both form azo dyes, but only the derivative of chlorpheniramine is suitable for photometric quantification on the thin-layer plate. The spots of codeine are also measurable, but the reproducibility is insufficient. The plasma levels are calculated by comparison with standards prepared with drug-free plasma.

For the purpose of this project, it was not necessary to identify the structures of the nitro and amino derivatives of codeine and chlorpheniramine produced by this procedure. We determined only the unchanged drugs and did not try to detect their metabolites.

EXPERIMENTAL

Reagents

A 10% solution of sodium hydroxide in distilled water and 1 *N* and 0.2 *N* hydrochloric acid solutions in distilled water were used.

The solvents chloroform, ethanol, ethyl acetate, diethyl ether (distilled), methanol, methylene chloride and 33% ammonia, were analytical grade.

Nitration mixture. To 40 ml methanol was added carefully with cooling a mixture of 30 ml of 65% nitric acid and 10 ml of 95–97% sulphuric acid. The mixture

of nitric and sulfuric acids was also prepared with great care and intensive cooling. The nitration mixture is stable over a period of weeks.

Titanium(III) chloride solution. To 20 ml of methanol were added 4 ml of titanium(III) chloride solution (15% solution in 4% hydrochloric acid). This mixture is stable only for 1 h.

Sodium nitrite solution. Immediately before use, 400 mg of sodium nitrite were dissolved in 20 ml of 1 *N* hydrochloric acid.

N-(1-Naphthyl)ethylenediamine solution. 1 g of N-(1-naphthyl)ethylenediamine dihydrochloride was dissolved in 100 ml of methanol. The solution is stable for some days.

Standards

Stock solutions. 20 mg of codeine phosphate·½ H_2O were dissolved in 50 ml of methanol (solution I). 10 mg of chlorpheniramine maleate were dissolved in 50 ml of methanol (solution II). 4 ml of solution II were diluted to 10 ml with methanol (solution III).

Standard solutions. 1 ml of solution I and 1 ml of solution III were mixed and diluted to 100 ml with chloroform (solution IV). 10 μl of this solution contain 40 ng of codeine phosphate and 8 ng of chlorpheniramine maleate. 5 ml of solution IV were placed in a 20-ml calibrated flask and made up to the mark with chloroform (solution V). 10 μl of this solution contain 8 ng of codeine phosphate and 2 ng of chlorpheniramine maleate.

Plasma standard solutions. In a 100-ml calibrated flask, 0.8 ml of solution I and 1 ml of solution III were mixed and made up to the mark with distilled water (solution VI). 1 ml of this solution contains 3.2 μg of codeine phosphate and 0.8 μg of chlorpheniramine maleate; 1 ml of solution VI was placed in a 20-ml calibrated flask and made up to the mark with plasma containing no substances which would cause interference (plasma I), 10 ml of plasma I were diluted to 20 ml with plasma (plasma II), 10 ml of plasma II were diluted to 20 ml with plasma (plasma III), 10 ml of plasma III were diluted to 20 ml with plasma (plasma IV). The plasma standards I–IV contain 160, 80, 40 and 20 ng/ml of codeine phosphate, and 40, 20, 10 and 5 ng/ml of chlorpheniramine maleate, respectively.

Material for TLC

Silica gel F_{254} 0.25-mm pre-coated thin-layer plates (Merck, Darmstadt, G.F.R.) were used. For application of the solutions to the thin-layer plates, the usual 10-μl micropipettes were used, *e.g.*, those of Dr. Barrolier. Chromatographic tanks having a capacity for 20-cm plates were employed for the development of the chromatograms. A Zeiss PMQ II chromatogram–spectral photometer was used as a scanner for the fluorescence and remission of the thin-layer plates.

Procedure

Extraction. Into 15-ml centrifuge tubes fitted with glass stoppers were placed 1 ml of the plasma to be analyzed or of the plasma standards I–IV, 0.5 ml of 10% sodium hydroxide solution and 7.5 ml of diethyl ether. Each mixture was extracted by shaking for 5 min on a reciprocating shaker, centrifuging for 5 min at 700 *g* and

transferring as much as possible of the ether layer into a 25-ml centrifuge tube fitted with a stopper. The extraction procedure was repeated with a second 7.5-ml portion of diethyl ether, centrifuged and the ether extracts were combined. 1 ml of 0.2 *N* hydrochloric acid was added to the combined ether extracts, which were then shaken for 5 min and centrifuged for 5 min at 700 *g*. The ether layer was carefully removed by aspiration without removing any of the acid phase. The acid extract was washed with 5 ml of diethyl ether, shaken for 5 min, centrifuged for 5 min and the diethyl ether was again removed by aspiration. The tube was heated for 2–3 min to *ca.* 60° in order to evaporate the ether residues. After cooling and addition of 0.5 ml of 10% sodium hydroxide solution and 5 ml of methylene chloride, the tube was shaken for 5 min and centrifuged for 5 min at 700 *g*. The aqueous phase was then aspirated (and discarded), and 4.5 ml of the organic phase were pipetted into a 10–15-ml tube. The tube was placed in a shaking thermostat at 55–60° until all of the organic solvent had evaporated, whereupon the tube was cooled and the residue was dissolved in 100 μl of chloroform.

Chromatography. The spots were applied 2 cm from the edges of the plate, the distance between the spots being 1.5 cm. In order to obtain small spots, the starting zone of the thin-layer plate was warmed to *ca.* 60° with a heating plate. The chloroform solution was transferred to the plate by use of a 10-μl micropipette, the tube was rinsed with 20 μl of chloroform and the rinsings were also applied to the plate. On the same plate were placed 10 μl of solutions I and II for the localization of codeine and chlorpheniramine after the development.

The chromatograms were developed twice in order to remove substances which might cause interference. The first eluent used was methanol, the development being carried out in a jar lined with filter-paper in order to achieve vapour saturation. The first elution distance was 12.5 cm. The plate was then dried for 5 min in an oven at 100°, cooled and placed in a second jar, lined with filter-paper, and pre-conditioned for exactly 5 min with the solvent system ethyl acetate–ethanol–33% ammonia (40:4:3). The plate was developed up to 10 cm, dried and the zone of codeine and chlorpheniramine was marked under short-wavelength UV light (254 nm).

Detection. (1) Fluorescence of codeine. The dry plate was sprayed with the nitration mixture until the layer was uniformly wet. When all the liquid had penetrated into the silica gel, the plate was placed in an oven at 110–115°. In order to achieve a high nitration grade, the thin-layer plate was placed on an aluminium block having a large heat capacity and which had been pre-heated in the oven, so that all of the chromatogram rapidly attained the oven temperature. Also important is a very intensive air circulation in the oven during the nitration. In order to drain off the acid vapours, a powerful water-jet pump was connected to the drying oven. After 10 min, the thin-layer plate was cooled to room temperature and the fluorescence of codeine was examined under long-wavelength UV light (366 nm).

The spots of codeine were quantified by measuring the fluorescence directly with a Zeiss PMQ II chromatogram–spectral photometer. The operating conditions are as follows: mercury lamp, excitation wavelength 300 nm; entrance diaphragm, 3.5 mm; slit width, 2 mm; filter, 578 nm; ordinate 4 times extended; damping, 2; scanning speed, 10 cm/min; paper speed, 10 cm/min.

(2) Colour reaction of chlorpheniramine. After scanning the fluorescence of codeine, the thin-layer plate was sprayed with the titanium(III) solution until the layer

was uniformly wet and then placed in a drying oven at 110–115° without ventilation. After 7 min the aromatic nitro compounds are reduced to the corresponding amino compounds. After cooling to room temperature, the chromatogram was sprayed with the sodium nitrite solution in hydrochloric acid and dried for 10 min with a cold stream of air. The chromatogram was sprayed with N-(1-naphthyl)ethylenediamine solution and after 1 min was dried with a stream of air at *ca.* 50–60°. Chlorpheniramine forms a purple azo dye; codeine shows a bluish grey colour.

The spots of chlorpheniramine were quantified with the Zeiss photometer under the following conditions: tungsten lamp, wavelength 550 nm; entrance diaphragm, 3.5 mm; slit width, 0.5 mm; ordinate four times extended; damping, 1; scanning speed, 10 cm/min; paper speed, 10 cm/min.

Calculation. With each series of samples, the four plasma standards (I–IV) were analyzed. The heights of the signals were directly proportional to the amount of the drugs applied to the plate. The height of the signals divided by the corresponding plasma concentration gave the corresponding height per ng/ml of plasma. The mean value of the four standards was compared with the height of the signal for the sample being analyzed

$$H_S/H_{PS} = \text{concentration of substances in plasma (ng/ml)}$$

where H_S is the height of the signal for the sample and H_{PS} is the mean height of the signals of the four plasma standards corresponding to a concentration of 1 ng/ml in plasma.

RESULTS AND DISCUSSION

Characteristics of the method

There was a linear relation between the amount and the height of the signals for codeine phosphate up to *ca.* 250 ng/ml, and for chlorpheniramine maleate up to *ca.* 60 ng/ml of plasma. For codeine, the sensitivity limit was 8 ng/ml of plasma, calculated as codeine phosphate, and for chlorpheniramine the limit was 1–2 ng/ml of plasma, calculated as chlorpheniramine maleate. By comparison of the signals of the substances applied directly to the thin-layer plate (standard solutions IV and V) with the signals of the substances extracted from plasma standards, it is possible to calculate the overall yield of the procedure. Taking into account the known aliquot loss, the recovery for both substances is *ca.* 90%.

With the described method, components of the plasma which caused interference could be eliminated. In blank plasmas, no substances were found to cause interference with codeine or chlorpheniramine. As already mentioned, I did not look for metabolites of both compounds, so that their possible co-determination cannot be excluded. Several plasma samples were analyzed twice on different days. Out of *ca.* 40 determinations, the relative standard deviation of the determination for codeine and chlorpheniramine was found to be ±6%.

Testing of the method in the determination of plasma levels of codeine and chlorpheniramine after therapeutic doses in man

With the assistance of Dr. Hennes, F. Hoffmann-La Roche, Grenzach, G.F.R.,

three test series were performed at 6–8-week intervals. Four volunteers each received one retard tablet containing 70 mg of codeine phosphate and 8 mg of chlorpheniramine maleate. Plasma samples were collected up to 48 h after medication (series B). Three of the same volunteers received, for 6 days every twelfth hour and on the seventh day in the morning, 1 retard tablet containing 70 mg of codeine phosphate and 8 mg of chlorpheniramine maleate. Plasma samples were obtained every 24 h before the next dose during the entire assay, and up to 120 h after the last administration.

The plasma levels measured in the three test series are summarized in Tables I–III and are plotted for one case in Fig. 2. The data for normal capsules agree with the values of Schmerzler *et al.*[1] and of Peets *et al.*[2]. After administration of the normal capsules to the four volunteers the plasma half-life for codeine was found to be 2.2–3.9 h. With the retard tablets it was not possible to determine a half-life, since the absorption of the drug and the elimination are superimposed. The minimum therapeutically effective plasma level for codeine is *ca.* 20 ng/ml calculated as codeine phosphate. Based on this figure, in the case of the two volunteers who received the same dose of codeine phosphate in normal capsules and in retard tablets, this level was reached with both formulations within *ca.* 0.5 h. When the retard tablets were administered, this plasma level was undercut 3–4 h later than with the normal capsules. On the other hand, it is obvious that in the case of the two other volunteers half of the dose in normal capsules produced nearly the same maximum plasma level as the whole dose in the retard tablet. The plasma half-life of chlorpheniramine in the four volunteers receiving the normal capsules lay between 24.5 and 36.3 h. These high values indicate that, in contrast to the codeine component of the combination, a retard formulation does not provide an advantage for the chlorpheniramine part. The plasma levels of this compound were similar after administration in normal capsules and in the retard tablets.

TABLE I

TEST SERIES A: PLASMA LEVELS (ng/ml) OF CODEINE AND CHLORPHENIRAMINE AFTER A SINGLE ORAL DOSE OF A RETARD TABLET CONTAINING 70 mg OF CODEINE PHOSPHATE AND 8 mg OF CHLORPHENIRAMINE MALEATE

a = Calculated as codeine phosphate, b = calculated as chlorpheniramine maleate.

Time after medication (h)	*Volunteer I*		*Volunteer II*		*Volunteer III*		*Volunteer IV*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.5	22	1.6	17	2.0	50	2.7	84	2.2
1.0	67	2.8	55	3.3	73	5.1	81	2.8
2.0	77	4.2	104	8.3	66	6.9	87	3.7
4.0	74	5.2	92	10.0	80	8.2	72	5.1
6.0	54	5.5	53	11.0	69	11.0	58	5.5
8.0	36	6.3	43	9.5	50	13.0	58	8.8
10.0	32	6.8	38	12.0	41	12.0	50	9.1
15.0	14	5.9	16	8.5	20	9.4	17	8.3
24.0	9	3.5	<8	7.2	12	13.0	11	5.6
32.5	<8	2.9	<8	5.3	8	11.0	<8	4.7
48.0	<8	2.1	<8	5.1	<8	6.0	<8	3.5

TABLE II

TEST SERIES B: PLASMA LEVELS (ng/ml) OF CODEINE AND CHLORPHENIRAMINE AFTER A SINGLE ORAL DOSE OF A NORMAL CAPSULE CONTAINING 70 mg OF CODEINE PHOSPHATE AND 8 mg OF CHLORPHENIRAMINE MALEATE (VOLUNTEERS I AND IV) OR 35 mg OF CODEINE PHOSPHATE AND 8 mg OF CHLORPHENIRAMINE MALEATE (VOLUNTEERS II AND III) RESPECTIVELY

For meaning of a and b see Table I.

Time after medication (h)	*Volunteer I*		*Volunteer II*		*Volunteer III*		*Volunteer IV*	
	a	*b*	*a*	*b*	*a*	*b*	*a*	*b*
0.5	177	3.1	9	2.6	43	1.5	85	1.7
1.0	253	4.4	11	3.4	91	10.0	226	4.8
2.0	200	6.3	96	4.2	61	20.0	198	10.0
4.0	124	7.4	67	14.0	36	20.0	106	10.0
6.0	66	8.9	36	13.0	29	23.0	65	10.0
8.0	31	12.0	19	12.0	8	15.0	41	11.0
10.0	16	9.6	—	—	<8	11.0	27	10.0
15.0	9	8.5	8	6.2	<8	8.5	15	11.0
24.0	<8	6.3	<8	6.2	<8	8.1	<8	4.5
32.5	<8	5.5	<8	3.8	<8	7.7	<8	3.6
48.0	<8	4.0	<8	3 0	<8	4 7	<8	2.4

TABLE III

TEST SERIES C: PLASMA LEVELS (ng/ml) OF CODEINE AND CHLORPHENIRAMINE AFTER MULTIPLE ADMINISTRATION OF A RETARD TABLET CONTAINING 70 mg OF CODEINE PHOSPHATE AND 8 mg OF CHLORPHENIRAMINE MALEATE

Medication times: each day at 8.00 and 20.00; first time on day 0, last time on day 6. For meaning of a and b see Table I.

Day and time of sampling		*Volunteer I*		*Volunteer III*		*Volunteer IV*	
		a	*b*	*a*	*b*	*a*	*b*
1	7.45	66	22	24	27	43	9.2
2	7.45	131	33	36	42	109	15
3	7.45	88	33	25	58	67	17
4	7.45	84	31	45	65	63	20
6	7.45	103	34	44	60	62	24
	10.00	146	41	117	59	162	27
	12.00	152	41	122	81	158	30
	16.00	76	44	90	51	87	24
	20.00	48	37	24	49	58	22
7	8.00	11	26	<8	46	9	20
8	8.00	<8	13	<8	36	<8	8.6
9	8.00	<8	7.3	<8	21	<8	3.9
10	8.00	<8	2.7	<8	12	—	—

CONCLUSIONS

The method described allows the determination of the plasma levels of codeine phosphate and chlorpheniramine maleate after therapeutic oral doses of the combined drugs. The procedure is highly sensitive and well reproducible.

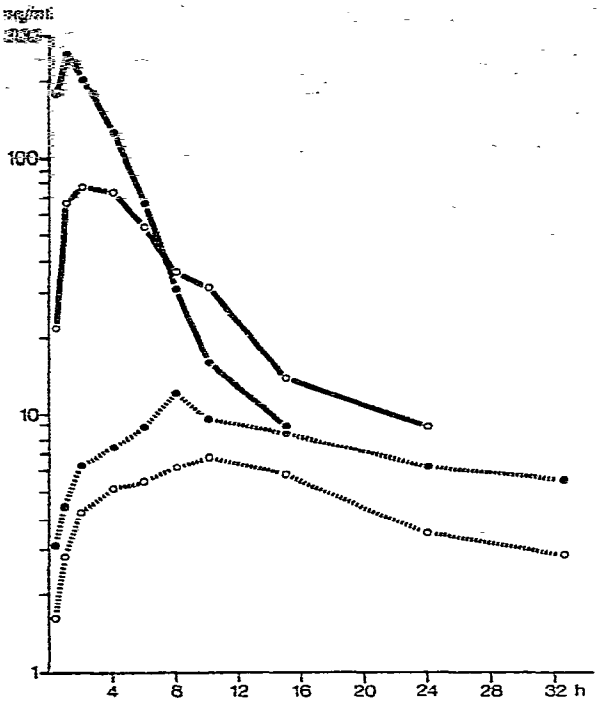


Fig. 2. Plasma levels of codeine and chlorpheniramine in volunteer I after a single dose of a retard tablet containing 70 mg of codeine phosphate and 8 mg of chlorpheniramine maleate (○—○, codeine phosphate; ○—○, chlorpheniramine maleate), or of a normal capsule containing 70 mg of codeine phosphate and 8 mg of chlorpheniramine maleate (●—●, codeine phosphate; ●—●, chlorpheniramine maleate).

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