

CHROMBIO. 470

Note

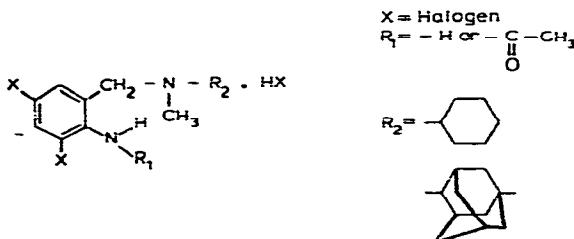
Determination of a new mucolytic drug Adamexina in biological liquids by photodensitometry

C. ALBET, M.G. SÁNCHEZ* and J. COLOMÉ

Analysis Department, Centro de Investigación Farmacéutica, Ferrer Internacional, S.A., c/ Juan de Sada 32, Barcelona-28 (Spain)

(First received July 23rd, 1979; revised manuscript received October 23rd, 1979)

The main clinical and pharmacological action of mucolytic drugs is that of modifying the bronchial secretion, lowering its viscosity and secretolytic action, increasing the fluidity of sputum and facilitating its expulsion, while lowering the coughing reflexes. The drug typifying this class of compounds is Bromexine, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)ammonium chloride, the properties of which have been described by several authors [1-8]. Investigations oriented towards obtaining a product with a wider field of action have resulted in the synthesis of Adamexina[®]*, in which the cyclohexyl ring of Bromexine is replaced by adamantane, which lends to the new mucolytic molecule its sympathicomimetic and antiviral properties [9-14].



Pharmacokinetic studies on Bromexine agree in pointing out the impossibility of determining levels in blood and urine by chemical methods, due to the low values found, so that some methods are proposed, based upon a labelled molecule, thus differentiating the intact drug from the several

*To whom correspondence should be addressed.

**Ferrer's Synthesis and Patent.

metabolites by chromatographic separation. Preliminary tests carried out with Adamexina show that the levels in plasma will also be low, probably due to the binding of the drug with blood cells and to its affinity with several organs and tissues as described for Bromexine [15, 16].

Attempts at conducting pharmacokinetic studies in human patients have resulted in the need for developing and improving the usual physico-chemical methods in spite of the above mentioned difficulties. Thus the drug is isolated from the remaining plasma components by a suitable extractive and chromatographic system, and then determined.

Adamexina shows UV absorption at 288 nm in ethanol, however $E_{1\text{ cm}}^{1\%} = 11.8$ is too low to make direct quantitative determinations. On the other hand, due to the molecular structure, formation of a fluorescent derivative is not easily attained and native fluorescence is zero. Separation by gas chromatography can be carried out in a silanized glass column packed with 3% OV-17 on Chromosorb W HP (100–120 mesh). However the maximum sensitivity obtained is only 50 ng because of adsorption problems making the quantitative analysis difficult.

A method yielding better results was that proposed by Haefelfinger [17, 18]. This method is based on the introduction of nitro groups into the aromatic ring followed by reduction to form the primary amine, diazotization and coupling, either forming a coloured compound or reacting the amine formed with fluorescamine to yield a fluorescent compound. All these reactions are carried out *in situ* on silica gel chromatoplates.

EXPERIMENTAL

Reagents

Anhydrous sodium sulphate, concentrated hydrochloric acid, 1 N potassium hydroxide, absolute ethanol, chloroform and diethyl ether were analytical grade.

Nitrating mixture. To 40 ml of methanol is carefully added a mixture of 30 ml 65% nitric acid and 10 ml 95–97% sulphuric acid, with continuous cooling. The mixture of nitric and sulphuric acids is very carefully prepared and under very intense cooling. This mixture is stable for several weeks.

Reducing titanium chloride solution. To 20 ml of methanol, 4 ml of titanium(III) chloride solution (15% of the titanium salt in 4% hydrochloric acid) are added. This solution is not stable for more than one hour.

Sodium nitrite solution. Sodium nitrite (400 mg) is dissolved in 20 ml of 1 N hydrochloric acid. It is prepared just before use.

N-(1-Naphthyl)ethylenediamine solution. N-(1-Naphthyl)ethylenediamine dihydrochloride (1 g) is dissolved in 100 ml methanol. Although this solution is stable for several days, it is preferable to prepare it just before use.

Standards

Adamexina (prepared by our Department of Synthesis) is dissolved in absolute ethanol, in various concentrations.

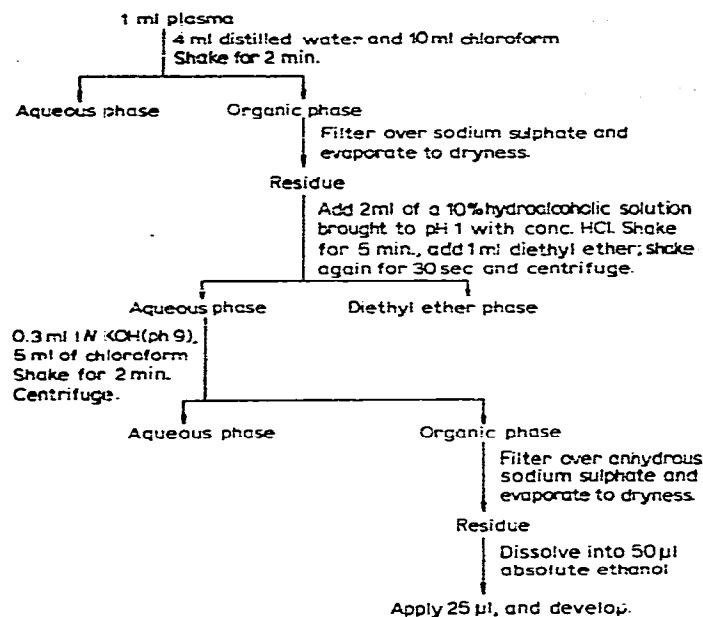
Thin-layer chromatography

Commercially available silica gel G plates (20 X 20 cm, 0.25 mm thick, Merck, Darmstadt, G.F.R.) without fluorescence are used. The tanks were obtained from Desaga (Heidelberg, G.F.R.). Micropipettes (20 or 25 μ l) for application of solutions onto the plates were obtained from Pedersen (Copenhagen, Denmark).

Apparatus

In order to quantify the spcts *in situ* a Zeiss PMQ II spectrophotodensitometer was used.

Extraction



Chromatographic conditions

Stationary phase: silica gel G activated for 45 min at 110°. Mobile phase: ethyl acetate-acetic acid-water (60:15:15). Elution height: 10 cm. Elution time: 1 h 15 min. R_F : 0.6-0.7. Chamber: not saturated. Elution temperature: room temperature (20-25°).

Detection

The detection consists of four steps:

(1) Nitration. Spray with sulphonitric mixture. Place the plate in a lidded stainless-steel box, which is kept in an oven at 115° for 15 min. Subsequently, it is cooled.

(2) Reduction. Spray with titanium chloride solution in methanol. Put in an oven at 115° for 30 min. Remove the plate and allow to cool.

(3) Diazotization. Spray with sodium nitrite dissolved in an acid medium. Then dry thoroughly with cold air.

(4) Coupling. Spray with N-(1-naphthyl)ethylenediamine solution in methanol. Then dry for 1 min under warm air. Slightly diffused, violet-rose spots appear.

Instrumental parameters

Apparatus: Zeiss PMQ II spectrophotodensitometer. Reading form: remission. λ_{max} : 510 nm. Slit: variable, according to concentration. Register speed: 50 mm/min. Scanning speed: 50 mm/min.

Calculations

The area unit means (A_m) obtained on reading the spots in both directions (from the elution front and perpendicular to it) and integration of the respective chromatographic peaks are used for standards in order to draw the corresponding calibration straight line, for each plate. Usually [19-25], the $A_m^2/100$ ratio and the quantity applied (μg) give the best coefficients of correlation and therefore are often used.

As readings are not referred to an absolute blank but to the plate blank, the straight lines do not pass through the origin. Nevertheless this is not important if working within the experimental range of the straight line and the line is not extrapolated. The obtained values, corrected with the dilution factors, will give the actual plasma concentration expressed in $\mu\text{g}/\text{ml}$.

RESULTS AND DISCUSSION

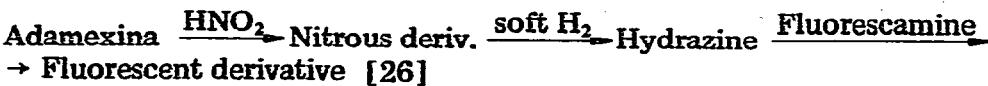
Extraction

The deproteinization of plasma has been attempted with the usual reagents, such as trichloroacetic acid and mixtures of sodium tungstate-maleic acid. In each case, although the precipitation is abundant, the precipitating agent reacts with Adamexina or, at best, secondary reactions take place with some of the other plasma components, giving a sequence of chromatographic spots which interfere with the estimation of the Adamexina.

Precipitation with organic solvents has been the only viable way tried with any success. With this in view, the most suitable solvent was chosen, through the calculation of the partition coefficients (Table I). Chloroform, because of the greater solubility of the drug at any pH, and diethyl ether, because of the slight solubility of the drug at acid pH, were the most advantageous solvents for the extraction and washing of the extracts, respectively.

Formation of derivatives

The procedures for obtaining derivatives *in situ* have been considered, and the following reactions appeared to be very sensitive:



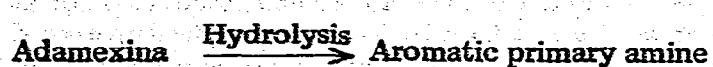
The reaction takes place, but it has little sensitivity. Detection limit is 250 ng/ml.

TABLE I

CALCULATION OF THE PARTITION COEFFICIENTS OF ADAMEXINA FOR DIFFERENT pH VALUES

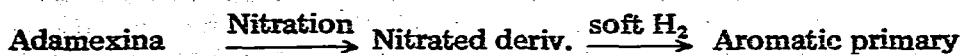
For a concentration of 25 mg in 100 ml of a 10% hydroalcoholic solution. Extraction is made with equal volumes.

Solvent	pH = 5.7 (water)		K_p	pH = 8 (NH_4OH)		K_p	pH = 9 (acetic acid)		K_p
	Aqueous phase	Organic phase		Aqueous phase	Organic phase		Aqueous phase	Organic phase	
Chloroform	0.085	0.385	77	0.020	0.380	19	0.040	0.385	9.1
Carbon tetrachloride	0.155	0.200	1.3	0.175	0.290	1.7	0.255	0.100	0.4
Diethyl ether	0.150	0.170	1.1	0.185	0.130	0.7	0.250	0.060	0.3
Benzene	0.140	0.215	1.5	0.145	0.210	1.5	0.230	0.115	0.5
Methylene chloride	0.020	0.395	19.8	0.040	0.365	9.1	0.070	0.320	4.6



- a) Diazotization and Coupling \rightarrow Coloured derivative
- b) Fluorescamine \rightarrow Fluorescent derivative

There are some difficulties in effecting the initial hydrolysis in a quantitative manner. Recovery is 60—70%.



- amine a) Diazotization and coupling \longrightarrow Coloured derivative
- b) Fluorescamine \longrightarrow Fluorescent derivative

(a) The formation of a coloured derivative, although requiring several successive treatments, does not present prohibitive complications. The optimal conditions have to be established by experiment, by trying different reagent concentrations, as well as times and temperatures for each of the steps of the reaction. In this respect, the colour of the background of the plate plays a decisive role. When the reaction rate is increased, especially the nitration and the reduction, the background colour rises rapidly.

Under the final conditions, the violet-rose colour of the spot, as well as the

TABLE II

EXAMPLES OF CALIBRATION STRAIGHT LINES FOR DIFFERENT CHROMATO-PLATES

Plate	Quantity applied (μg)	$\text{Am}^2/100$
1*	1	100
		109
	0.5	52
		57
	0.1	11
		15
2**	1	105
		114
	0.5	58
		56
	0.1	18
		17
3***	2	424
		394
	1	181
		182
	0.5	56
		48

$$*r = 0.99; y = 2.90 + 101.6x.$$

$$**r = 0.99; y = 6.75 + 102.3x.$$

$$***r = 0.99; y = 61.75 + 236.5x.$$

yellowish white hue of the background of the plate remain stabilized for at least 5 h; this time can be increased up to 24 h by keeping the plate protected from light. The sensitivity limit, in this case, is 50 ng, which is sufficient for our purpose. The reaction can also be carried out with Bromexine or with compounds having similar structures, although in this specific case the sensitivity is only 250 ng, a limit which can doubtlessly be improved by a specific study of every step of the reaction.

The response of the blank plasma is nil in human plasma and in rat plasma when it is fresh, and there appears only a very slight spot at the same R_F as Adamexina when these plasma samples have been kept for some days in a freezer; but even at worst the interference would only represent 20 ng, that is to say, out of our sensitivity limits.

(b) Owing to the positive evolution of the above test, it has not been deemed necessary to consider this possibility.

Calibration curve

Due to the multiple factors involving the chromatographic process, especially in the development step, it is necessary to draw a calibration straight line independently for each chromatoplate in order to attain a fair adjustment. Several examples are shown in Table II.

TABLE III
VALUES OF THE PERCENTAGES OF RECOVERY IN PLASMA

The range of concentration was 0.5–4 $\mu\text{g}/\text{ml}$ equivalent to 0.25–2 μg applied onto the plate. Mean percentage = 82.4%; S.D. = 6.78; n = 20; variation coefficient ($P = 95\%$) = 3.2%. Straight line parameters: $r = 0.9902$; $y = 0.0517 + 0.7421x$.

Quantity applied (μg)	Recovery (%)
2	75
	85
	70
	70
	83
1	78
	75
	80
	85
0.5	85
	92
	85
	85
	78
	80
	92
0.25	88
	90
	92
	80

Recovery and confidence limits

The mean percentage of recovery in plasma is 82.4% with a range of concentrations between 0.25 and 2 µg applied on the plate.

The statistical data needed to establish the precision of the method and to calculate the corresponding calibration straight line are given in Table III.

In conclusion, the method is suitable for the estimation of levels of Adamexina and of other 2-amino-3,5-dibromobenzyl derivatives in plasma; the pharmacokinetics of such drugs in man or in some animal species, as well as the bioavailability of the various pharmaceutical preparations, will be studied further.

REFERENCES

- 1 R. Engelhorn and S. Püschman, *Arzneim. Forsch.*, 13 (1963) 474.
- 2 H.J. Merker., *Arzneim.-Forsch.*, 16 (1966) 509.
- 3 H. Eigelsreiter and M. Mair, *Arzneim.-Forsch.*, 17 (1967) 353.
- 4 F. Arch, *Arzneim.-Forsch.*, 13 (1963) 480.
- 5 H. Bürgi, *Schweiz. Med. Wochenschr.*, 95 (1965) 274.
- 6 W. Guenthner, *Fortschr. Med.*, 83 (1965) 701.
- 7 F. Krug, *Forsch. Prax. Fortb. (Med.)*, 17 (1966) 99.
- 8 R. Giesecking and R. Baldamus, *Beitr. Klin. Tuberk. Spezifischen Tuberk. Forsch.*, 137 (1968) 1.
- 9 R.R. Grunert, J.W. McGahen and W.L. Davies, *Fed Proc., Fed. Amer. Soc. Exp. Biol.*, 23 (1964) 387.
- 10 W.L. Davies, R.R. Grunert, R.F. Haff, J.W. Mc Gahen, E.M. Neumayer, M. Paulschock, J.C. Watts, T.R. Wood, E.C. Hermann and C.E. Hoffman, *Science*, 144 (1964) 862.
- 11 G.G. Jackson, R.L. Muldoon and L. Alkers, in J.C. Sylvester (Editor), *Proceedings of the Third Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington, D.C., 1963, Am. Soc. Microbiol., Ann Arbor, Mich., 1964, p. 703.
- 12 H.A. Wendel, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 23 (1964) 387.
- 13 K.W. Cochran and H.F. Maassab, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 23 (1964) 387.
- 14 H.F. Maassab and K.W. Cochran, *Science*, 145 (1964) 1443.
- 15 G. Von Beisenheiz, *Ther. Umsch.*, 22 (1965) 322.
- 16 R. Jauch and R. Hankwitz, *Arzneim.-Forsch.*, 25 (1975) 12.
- 17 P. Haefelfinger, *J. Chromatogr.*, 111 (1975) 323.
- 18 P. Haefelfinger, *J. Chromatogr.*, 124 (1976) 351.
- 19 P. Kubelka and F. Munk, *Z. Tech. Phys.*, 12 (1931) 593.
- 20 K. Schunack, *Arzneim.-Forsch.*, 19 (1969) 95.
- 21 H. Zürcher, G. Pataki, J. Borko and R.W. Frei, *J. Chromatogr.*, 43 (1969) 457.
- 22 R.W. Frei, H. Zürcher and G. Pataki, *J. Chromatogr.*, 45 (1969) 284.
- 23 G. Pataki, *Chromatographia*, (1968) 1492.
- 24 S. Schilcher, *Sci. Pharm.*, 39 (1971) 151.
- 25 H. Jork, *Z. Anal. Chem.*, 221 (1966) 17.
- 26 J.C. Young, *J. Chromatogr.*, 124 (1976) 17.