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RAPID THIN-LAYER CHROMATOGRAPHIC PHOTODENSITOMETRIC METHOD FOR THE DETERMINATION OF METOCLOPRAMIDE AND CLEBOPRIDE IN THE PRESENCE OF SOME OF THEIR METABOLIC PRODUCTS

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SUMMARY

Metoclopramide and its newly developed analogue clebopride, together with some of their metabolic products are quantitated, following extraction from biological tissues and fluids, and subsequent separation on silica gel thin-layer chromatographic plates. Diazotisation, followed by coupling with N-(1-naphthyl)ethylenediammonium dichloride, carried out on the thin-layer plate, is utilised for visualisation. The intensity of the spots is measured by photodensitometric analysis. The effect of variation of various experimental conditions is studied. The method has proven to be satisfactory for the measurement of 20 ng/ml of these compounds in biological material; the results are well within the accepted limits of deviation.

INTRODUCTION

Metoclopramide (I, Primperan®, Maxolon®), a benzamide, is an anti-emetic drug[1–3], clebopride (II, Cleboril®) is a recently developed, more potent analogue[4,5]. On metabolism, I produces III[6–9], IV[9,10], V[6,10] and VI[8,9], while II is metabolised to produce V[11], VII[12] and VIII[12] (see Table I for structures). Although I is metabolised by O-demethylation[9,10], so far, attempts to identify IX as a metabolic product of II have not been successful; yet it was included in the present study.

High polarity and low volatility preclude a routine gas–liquid chromatographic (GLC) analysis of all compounds I–IX. General ultraviolet or colorimetric

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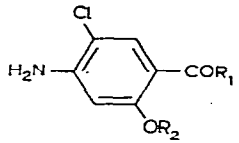
TABLE I

STRUCTURES AND TLC R_F VALUES OF METOCLOPRAMIDE (I), CLEBOPRIDE (II) AND OF SOME OF THEIR METABOLIC PRODUCTS

Bz = benzyl; n.d. = not determined.



Comp. no.	*Substituents*		*R_F in solvent systems 1–4*			
	R_1	R_2	*1*	*2*	*3*	*4*
I	$-NH(CH_2)_2N(C_2H_5)_2$	CH_3	0.10	0.46	0.19	0.54
II	$-NHCH(CH_2)_2N(Bz)CH_2CH_2$	CH_3	0.61	0.65	0.56	0.63
III	$-NH(CH_2)_2NHC_2H_5$	CH_3	0.05	0.19	0.28	0.25
IV	$-NH(CH_2)_2NH_2$	CH_3	0.02	0.11	0.31	0.20
V	$-OH$	CH_3	0.47	0.01	0.72	0.15
VI	$-NHCH_2CH_2OH$	CH_3	0.45	0.27	0.63	0.57
VII	$-NHCH(CH_2)_2NHCH_2CH_2$	CH_3	0.02	0.07	0.40	0.10
VIII	$-NHCH(CH_2)_2N(O)(Bz)CH_2CH_2$	CH_3	0.19	0.19	0.62	0.41
IX	$-NHCH(CH_2)_2N(Bz)CH_2CH_2$	H	n.d.	0.47	0.64	0.53

methods do not allow simultaneous determination[6,13,14]. In addition, since the pK_a values of I, II, III, IV and VII are all within the range 7.6 (for II)–9.3 (for VII), selective extraction procedures, based on differences in pH alone, cannot be applied[11]. Recently, one of us used the diminution of background fluorescence at 254 nm on silica gel F_{254} plates to obtain pharmacokinetic data for I (quantitative) and III (semi-quantitative)[7]. Also, high-performance liquid chromatography (HPLC) has been used for quantitation of I, although it involves elaborate procedures[9]. In addition, separation using HPLC of compounds I–IX has not been obtained.

In the present study, increased sensitivity and selectivity was obtained, using an *in situ* diazo-coupling technique.

High precision in thin-layer chromatographic (TLC) photodensitometry can be obtained only when all critical procedures involved are regidly standardised[15]; in the present study attempts have been made to eliminate variation in the analysis caused by differences in the techniques of spot application, in plate development, diazotisation and coupling, and in the use of various instrumental conditions.

MATERIALS AND METHODS

Compounds and materials

Compounds I (metoclopramide; hydrochloride), III (free base), IV (free base) and VI were kindly supplied by Beecham Pharmaceuticals (Brentford, Great Britain) and compounds II (clebopride; HCl salt and hydrogen maleate), V, VII (free base and hydrogen fumarate), VIII and IX (free base) by Laboratorios Almirall (Barcelona, Spain). The purity of these compounds was checked by TLC, GLC, HPLC, nuclear

magnetic resonance or mass spectrometry. Sodium nitrite and N-(1-naphthyl)ethylenediammonium dichloride were purchased from BDH (Poole, Great Britain) and Merck (Darmstadt, G.F.R.), respectively. All organic solvents were freshly distilled (twice), not more than 24 h before use.

The spots were applied on the TLC plates, using capillary pipettes (E. C. Linskey, London, Great Britain) or Hamilton micro-syringes of 5-, 10- or 100-μl capacity. Glass TLC plates (5 × 20 cm and 20 × 20 cm), precoated with 0.25 mm of silica gel 60 F_{254}, were purchased from Merck. Rectangular chromatographic tanks (7.5 × 23 × 23 cm), backed with Whatman No. I filter paper, were used for development of the plates.

The sources of co-factors and the composition of buffers used are described elsewhere[8].

Thin-layer chromatography

The TLC plates were activated for 1 h at 110° before use. The spots were applied under a stream of air at room temperature; the centres of the spots were 2 cm apart and at least 2.5 cm from the edge of the plate.

The compositions of the TLC solvent systems were: methanol–chloroform (1:4) (solvent 1), 1,2-dichloroethane–ethanol–ammonia solution (sp. gr. 0.88) (70:15:2) (solvent 2), *n*-butanol–acetic acid–water (4:1:1) (solvent 3) and isopropanol–ammonia solution (sp. gr. 0.88)–water (80:4:5) (solvent 4); the components were thoroughly mixed and the mixture was put in the TLC tank 1 h before use. Spraying of the plates was carried out from approximately 30 cm distance, in zig-zag movements, covering the whole of the appropriate side of the plate evenly, over a period of 20 sec per spray solution per plate of 20 × 20 cm. The plates were dried in a stream of air at room temperature after development and after each spray. For storage after photodensitometric scanning, the plates were covered with 20 × 20 cm glass plates and the edges sealed with Sellotape. The spots, protected in this manner, are stable for at least some months (crude observation).

Apparatus

Photodensitometric measurements were made using a "Chromoscan" (Joyce, Loebl & Co., Gateshead, Great Britain), in conjunction with a Joyce Loebl thin-layer scanner, employing the recording and integrating facilities of the parent instrument. Quartz optics were fitted. Scanning speed was approximately 2 mm/sec. The instrument was used in the reflectance mode.

For measurements of diminution of background fluorescence, a Camag-Turner T scanner (Camag, Muttenz, Switzerland) was used (filter 810 as primary and 823 as secondary; slit No. 15; aperture No. 16; scanning speed 600 mm/h), equipped with a Servoscribe IS recorder.

The TLC plates were sprayed using a chromatographic sprayer (Shandon Products, Cheshire, Great Britain). Other equipment included an airblower (not heated), a bench centrifuge, a film evaporator, a mechanical shaker and long (350 nm) and short (254 nm) ultraviolet lamps (Camag, Cambridge, Great Britain).

Incubations

Details of the preparation of the liver fractions and of the incubation procedures are given elsewhere[8].

Effect of variation of HCl normality of the diazotisation spray solution on the photodensitometric detector response

Of a solution of I in methanol, aliquot parts of 20 μl (1 nmole) were spotted on TLC plates. The plates were developed in solvent system 1. The different spots were then sprayed with freshly prepared solutions of 1.0% (w/v) sodium nitrite in hydrochloric acid of a normality ranging from 0 to 10 *N*. After drying, the plates were sprayed with a 0.4% (w/v) solution of N-(1-naphthyl)ethylenediammonium dichloride in methanol.

Photodensitometry was carried out using optimum filters (*i.e.* 550 nm) and aperture dimensions (*i.e.* 5 × 0.5 mm).

Effect of variation of concentration of N-(1-naphthyl)ethylenediammonium dichloride of the coupling spray solution on the photodensitometric detector response

Of a solution of I in methanol, aliquot parts of 20 μl (1 nmole) were spotted on TLC plates. The plates were developed in solvent system 1 and were then sprayed with a freshly prepared 1.0% (w/v) solution of sodium nitrite in 1 *N* hydrochloric acid. After drying, the different spots were sprayed with solutions of N-(1-naphthyl)-ethylenediammonium dichloride in methanol, ranging in concentration from 0 to 2.0% (w/v).

Photodensitometry was carried out, using optimum filters (550 nm) and aperture demensions (5 × 0.5 mm).

Effect of variation of the filter wavelength on the photodensitometric detector response

Of a solution of I in methanol, 20 μl (1 nmole) was spotted on a TLC plate. Diazo-coupling was carried out using spray solutions of optimal strength (*i.e.* 1 *N* HCl for diazotisation and 0.4% (w/v) coupling reagent for coupling). Photodensitometric scanning was carried out using filters of different wavelength, and an aperture slit of 5 × 0.5 mm.

Effect of variation of the aperture slit dimensions on the photodensitometric detector response

The above procedure was followed, except that photodensitometric scanning was carried out, using aperture slits of different dimension and a filter of 550 nm.

Precision

To obtain information concerning the precision of the scanning procedure, of a solution of I in methanol, 20 μl (1 nmole) was spotted on a TLC plate. Diazo-coupling was carried out using spray solutions of optimum strength (*i.e.* 1 *N* HCl for diazotisation and 0.4% (w/v) coupling reagent for coupling). Repetitive photodensitometric scanning ($n = 9$) was carried out using optimum filters and aperture. The readings were computed to obtain the values for standard error (SE), standard deviation (SD) and coefficient of variation (CV).

Data concerning the precision of photodensitometric scanning plus spot application plus diazo-coupling were obtained using the above procedure, except that nine spots, of 5 nmole each, were applied and that after diazo-coupling each spot was scanned only once.

Effect of travel distance on silica gel on the photodensitometric detector response

Of a solution of II (hydrogen maleate) in methanol, aliquot parts of 20 μl (1 nmole) were spotted on 5 × 20 cm TLC plates. The plates were developed in solvent system 1 for different times, to obtain plates with solvent fronts of 9–18 cm from the baseline. Diazo-coupling and photodensitometric analysis were carried out, using the optimum conditions described above.

Linearity of calibration curves

Of solutions in methanol of I–IX, different amounts (ranging from 0.1 to 5.0 nmole) were spotted on TLC plates. The plates were developed in the appropriate solvent systems, and diazo-coupling followed by photodensitometry was carried out, using optimum conditions. I and II were applied, each in the presence of their main metabolic products and/or reference compounds.

The data were plotted and computed to obtain the correlation coefficients.

Extractability

Contents of stoppered tubes, containing 6-ml solutions (in phosphate buffer) of 25 nmole I and II, each in the presence of 25 nmole of their respective metabolic products or reference compounds, were adjusted to pH values of 2.0, 7.4 and 13.0, using 2 *N* HCl and 2 *N* NaOH. After shaking for 30 min with 15.0 ml chloroform, the layers in each tube were separated by centrifuging. Of each chloroform phase, 10.0 ml was taken, and evaporated at 50° under reduced pressure. The residue was dissolved in 100.0 μl of methanol, and 20.0 μl was applied on TLC plates. Development of the plates was carried out in the appropriate solvent systems. Diazo-coupling and photodensitometric analysis was carried out, using the optimum conditions described above.

The extractability from spiked liver homogenates was studied using the above procedure, except that solutions of I–IX in liver homogenates were used, instead of in phosphate buffer.

To obtain information concerning its practical use, the present procedure was applied to incubation mixtures of II with 9000 *g* supernatant of liver homogenates of male NZW rabbits.

RESULTS AND DISCUSSION

The formation of diazo-compounds in situ

Since diazotisation of primary aromatic amines is an exothermic reaction[16], spraying of the TLC plates with $NaNO_2$–HCl, and subsequent drying of the plates, is not carried out at elevated temperature; room temperature was used in the present studies. The stability of these aryl-diazonium salts is indicated by the small degree of breakdown (approx. 20% for a spot containing 1 nmole of compound I) that occurred when the sprayed plates were heated for 10 min at 70°.

Diazotisation in solution must normally be carried out under conditions of fairly high acidity[17]. Similarly, in the present study, where the diazotisation reaction is carried out on a silica gel surface, the HCl normality of the spray solution for diazotisation has to be greater than 1 *N* to obtain a maximal photodensitometric detector response (Table II). This table also shows that there is apparently no maximum acidity for this reaction.

TABLE II

EFFECT OF VARIATION OF NORMALITY OF HCl OF THE SPRAY SOLUTION FOR DIAZOTISATION AND OF VARIATION OF CONCENTRATION OF N-(1-NAPHTHYL)-ETHYLENEDIAMMONIUM DICHLORIDE OF THE SPRAY SOLUTION FOR COUPLING ON THE PHOTODENSITOMETRIC DETECTOR RESPONSE, FOR A SPOT OF METOCLOPRAMIDE ON A SILICA GEL 60 F_{254} THIN-LAYER PLATE

PDR = photodensitometric detector response; NED = N-(1-naphthyl)ethylenediammonium dichloride; for all measurements, $n = 3$.

Normality HCl	*PDR (%)*	*Conc. NED (%, w/v)*	*PDR (%)*
0	12	0	0
0.001	17	0.005	54
0.01	28	0.01	59
0.1	47	0.02	85
0.5	87	0.05	100
1.0	97	0.075	97
2.5	100	0.5	98
5.0	98	1	97
10.0	100	2	100

Diazonium salts in general are only very weak electrophiles and will normally only attack highly reactive aromatic compounds[16]; the compounds in the present investigation contain electron-withdrawing groups *ortho* (*i.e.* chlorine) and *para* (*i.e.* carbonyl), enhancing their electrophilic character by increasing the degree of positive charge on the diazo-group.

The coupling reagent N-(1-naphthyl)ethylenediammonium dichloride contains an aromatic ring, activated by a powerfully electron-releasing group (*i.e.* –NH–R) in the *para* position. Diazo-coupling occurs instantly on spraying of the plates containing the diazotised amines with the coupling reagent in methanol, at room temperature. A concentration of 0.4% was selected for routine analysis (Table II). In theory, a concentration even below 0.04%, combined with a longer spraying time would give similar results, but then the silica gel surface becomes too "wet" and blurring occurs. The purple-red spots are stable at room temperature, when protected (see Materials and methods) and are not noticeably affected by storage at 70°, for at least 2 h. A slight excess of nitrous acid, known to interfere with the reaction of diazonium salts in solution[16] does not affect the above reaction; consequently treatment with ammonium sulfamate[18] or urea[16] was not carried out.

The effect of variation of some instrumental conditions on the photodensitometric detector response

When the plates, containing the diazo-coupled spots, were subjected to photodensitometric analysis, a maximum detector response was observed when the filter of 550 nm was used (Table III). The first report about a diazotised aromatic amine coupled with N-(1-naphthyl)ethylenediammonium dichloride mentions a maximum absorption at 545 nm, in solution[18]. Most workers use filters of approximately 540 nm, when analysing this type of compound colorimetrically[19,20].

The aperture slit of 5 × 0.3 mm gave the highest reading, for those concentrations relevant to metabolic work (Table III). Consequently, a filter of 550 nm and an aperture slit of 5 × 0.3 mm were selected for routine analysis. This newly estab-

TABLE III

EFFECT OF VARIATION OF FILTER WAVELENGTH AND OF VARIATION OF APERTURE SLIT DIMENSION ON THE PHOTODENSITOMETRIC DETECTOR RESPONSE, FOR A SPOT OF METOCLOPRAMIDE ON A SILICA GEL 60 F_{254} THIN-LAYER PLATE, AFTER DIAZO-COUPLING *IN SITU*

PDR = photodensitometric detector response.

Filter wavelength (nm)	*PDR (%)*	*Aperture size (mm)*	*PDR (%)*
465	43	5 × 0.3	100
490	66	5 × 0.5	92
520	75	10 × 0.3	81
550	100	10 × 0.5	76
620	71	10 × 1.0	72

lished method was compared with a method[7,21] where the diminution of background fluorescence of silica gel 60 F_{254} thin-layer plates, containing underivatised material, is measured. Scans were made of a TLC plate, developed in solvent system 3, containing an extract of an incubation mixture of I with a liver homogenate made alkaline before extraction.

The advantages of the diazo-coupling method over the fluorescence method were greater sensitivity (the detection limits per spot were 20 and 80 ng, respectively) and less interference (*e.g.* the huge TLC spot of nicotinamide caused no interference on photodensitometric analysis). In addition, scanning by the fluorescence method of the area containing the TLC spot of metabolically produced VI caused an unexpected negative detector response, possibly due to an extracted endogenous fluorescent impurity.

Precision of the method

The good reproducibility of the scanning of one spot, containing 1 nmole diazo-coupled I on a TLC plate is reflected in the low value for the standard deviation (A, Table IV). Also, spot application and the technique of diazo-coupling *in situ* are reproducible, although the standard deviation of scans of nine different spots, each containing 1 nmole of metoclopramide on silica gel, is of course somewhat greater (B, Table IV).

A much higher SD for spot application and/or visualisation would have necessitated the use of an internal (*i.e.* in the solution that is to be applied) marker. Another reason for not using a marker is the fact that this quantitative technique was developed for use in metabolic work; for an added marker, there is not enough space left on the TLC plate, already containing I or II, together with their respective metabolic products[11].

The precision of the whole analytical procedure is reflected in the values under C, Table IV.

Linearity of detector response with concentration

Linear calibration curves for all the compounds were obtained at concentration levels up to approx. 3 nmole (depending on the R_F value).

Regression analysis gave correlation coefficients of no less than 0.978 for calibration curves of six points (Table V). However, photodensitometric analysis with

TABLE IV

PRECISION OF PHOTODENSITOMETRIC SCANNING (A), OF SPOT APPLICATION (B) AND OF THE WHOLE ANALYTICAL PROCEDURE, INCLUDING EXTRACTION AT pH 12 (C) FOR METOCLOPRAMIDE

1 nmole of I was applied; solvent 2 was used.

Number of analysis	*Integration readings*		
	A	*B*	*C*
1	86	86	65
2	85	85	60
3	87	83	70
4	85	88	68
5	86	89	65
6	87	89	68
7	85	84	62
8	86	84	66
9	87	86	66
Mean	86.0	86.0	65.6
SE	0.3	0.7	1.0
SD	0.9	2.2	3.1
CV (%)	1.0	2.6	4.7

reasonable accuracy is possible at higher concentration levels, where calibration curves are no longer linear[22].

Effect of travel distance

In an attempt to simulate the effect of the R_F value on the integration reading (*i.e.* spot size and spot density), TLC plates, containing 0.5 nmole of II (*i.e.* the most lipophilic compound of the series) in spots on the baseline, were developed for different lengths of time and subsequently sprayed and analysed. Diffuse spots at the baseline gave higher readings than spots that had travelled up to a few centimetres (Fig. 1); however, beyond a travelling distance of 8 cm the spots became larger again, but without undue spreading, resulting in a higher integration reading. For major lipid classes, it has been reported[23] that peak area increases with R_F until a value of

TABLE V

LINEARITY OF PHOTODENSITOMETRIC DETECTOR RESPONSE WITH CONCENTRATION, AFTER DIAZO-COUPLING *IN SITU*

Compound	*Range used (nmole per spot; 6 points)*	*TLC solvent system*	*Correlation coefficient*
I	0.177–1.062	2	0.995
II	0.113–0.675	2	0.998
III	0.197–1.182	2	0.994
IV	0.270–1.617	2	0.999
V	0.163–0.975	1	0.978
VI	0.131–0.786	2	0.986
VII	0.104–0.623	2	0.999
VIII	0.110–0.657	2	0.996
IX	0.169–1.014	2	1.000

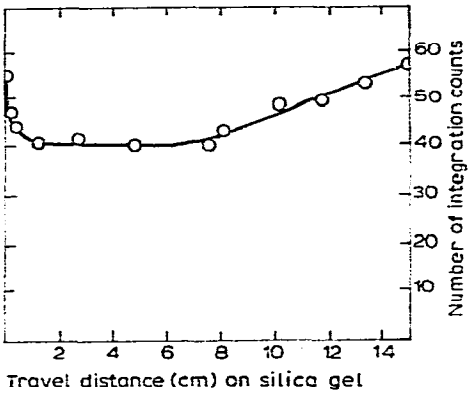


Fig. 1. Effect of travel distance on silica gel 60 F_{254} on the photodensitometric detector response, after diazo-coupling, for a spot containing clebopride, using solvent system 1.

0.4, remains constant between R_F values of 0.4 and 0.8, and increases again with R_F, beyond the value of 0.8.

However, since this applies to different compounds in one solvent system, it gives no clear picture of the effect of travelling distance for one compound on a TLC plate.

When spots containing Sudan Red G were allowed to travel different distances in one solvent system, the peak area varied less with distance of development than with time of contact of the solute with the mobile phase[24].

When the present method is used, complicated correction procedures for R_F values, based on thermodynamic principles[25] are not needed, unless authentic reference material is not available.

Extractability from different aqueous systems

The acidic, neutral and basic properties of the respective compounds in this study cause a great difference in extractability at the various pH values.

Maximum extraction from aqueous phase into chloroform clearly occurs at alkaline pH for compounds I, II, III, IV and VII (Table VI), since all of these compounds contain a basic centre. As expected, compound V is extracted only at acidic pH, because of its acidic character. The two compounds of neutral character in these series, *viz.* VI and VIII are not affected much in their extractability by pH changes, whereas the phenolic base IX shows incomplete extraction at a high pH value.

The difference in recovery from phosphate buffer (columns A, Table VI), and 9000 *g* supernatant (columns B, Table VI) necessitates the use of calibration curves, obtained from biological material, when accurate quantitative analysis is to be carried out.

When the compounds were extracted from the 9000 *g* soluble or microsomal fractions of liver homogenates, from urine or from blood, the extracted constituents of the biological tissues or fluids did not interfere with the analysis.

None of these constituents reacted visibly with the spray reagents, and the coloured constituents (some yellow–brown impurities were extracted from biological material at acidic pH) had R_F values that were different from those of the compounds under investigation.

TABLE VI

RECOVERY FROM PHOSPHATE BUFFER (A) AND FROM FORTIFIED 9000 *g* SUPERNATANT OF LIVER HOMOGENATES OF MALE NZW RABBITS (B) AT ACIDIC, PHYSIOLOGICAL AND ALKALINE pH VALUES

n.d. = not determined; results are expressed as mean values, $n = 2$; solvent for extraction: chloroform.

Compound 1	*TLC solvent 2*	*Percentage extracted (max. for each comp. = 100%)*					
		pH 2.0		*pH 7.4*		*pH 13.0*	
		A	*B*	*A*	*B*	*A*	*B*
I	2	2	n.d.	80	n.d.	100	90
II	2	24	n.d.	70	74	100	92
III	2	0	n.d.	53	n.d.	100	94
IV	2	0	n.d.	19	n.d.	100	93
V	1	100	90	14	7	0	0
VI	2	100	n.d.	94	94	97	90
VII	2	1	n.d.	54	60	100	94
VIII	2	96	n.d.	100	91	98	n.d.
IX	2	0	n.d.	100	n.d.	38	n.d.

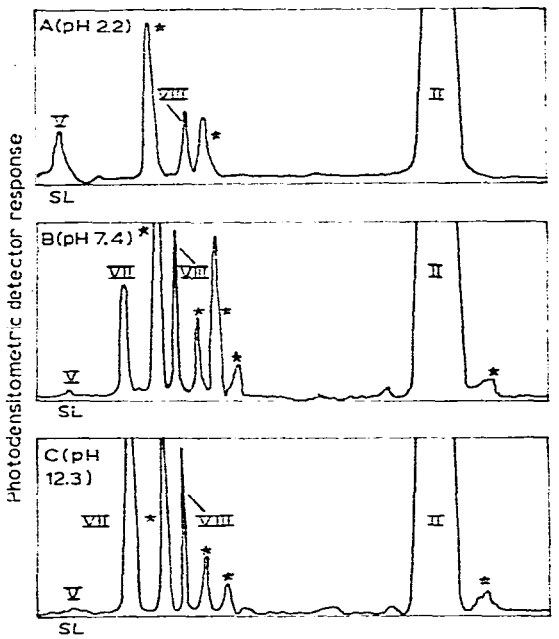


Fig. 2. Tracings of photodensitometric scans, after diazo-coupling, of concentrated chloroform extracts, obtained at pH 2.2 (A), pH 7.4 (B) and at pH 12.3 (C) of fortified incubation mixture (incubation time 60 min) of cleobpride with 9000 *g* supernatant of liver homogenates of male NZW rabbits, after ascending development in solvent system 2 over 20 cm on silica gel 60 F_{254} (SL = starting line; * = metabolic product of which no reference was available[11]).

An illustration of the quantitative use of this TLC photodensitometric method is given in Fig. 2, where tracings are shown of scans, after diazo-coupling, of incubation mixtures of clebopride (II) with fortified male NZW rabbit liver 9000 *g* homogenate. The difference in peak height of the various metabolic products, when extracted at different pH, reveals important physico-chemical information about the various products.

The quantitative analytical method described in this chapter combines the selectivity of TLC in appropriate solvent systems, of extraction procedures carried out at suitable pH values and of a sensitive diazo-coupling reaction, with the speed of a photodensitometric scanning procedure.

We conclude that the presented method is applicable to metabolic studies of metoclopramide and clebopride, and that the results are well within the accepted limits of deviation.

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