Journal of Chromatography, 130 (1977) 209–228

CHROM. 9352

THIN-LAYER CHROMATOGRAPHIC PROCEDURE FOR THE DETECTION, ISOLATION AND IDENTIFICATION OF BASIC PSYCHOTROPIC DRUGS IN URINE

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(Received May 18th, 1976)

SUMMARY

A procedure is described for the detection and identification of basic psychoactive drugs in urine. In the analytical system proposed, detection is dependent on thin-layer chromatography and chromophoric spraying of the resulting chromatogram. Identification is based on the extractability of the compound at the pH applied, on R_F values in two solvents, on colour characteristics after exposure to a sequence of reagents, on fluorescence and UV-absorption characteristics, on retention times in different gas–liquid chromatographic systems and on the behaviour in ion-pair extraction with methyl orange. The procedure has been applied to some narcotic alkaloids and amines which exhibit central stimulant action.

INTRODUCTION

An impressive volume of data has been accumulated on the behaviour of various psychoactive drugs in different physicochemical systems, and several analytical systems have been designed for the detection of these compounds in body fluids. The preferred procedure is organic solvent extraction, or extraction on a resin, followed by thin-layer chromatography (TLC) and visualization of the substance by spraying with a number of chromophoric reagents[1–9]. This approach is suitable for screening purposes, but is of restricted use in identification (*e.g.*, ref. 10). In view of the lack of specificity of TLC R_F values, even the use of multiple TLC systems[11–14] for identification may not be helpful. The combination of TLC with specific chromophoric spraying increases the specificity of the TLC procedure. However, the colour of a compound after TLC and spraying is also generally dependent on the concentration of substance in the sample, on the mode of application of the spray reagent and on the amount of reagent used for spraying.

The combination of TLC and gas–liquid chromatography (GLC) offers better conditions for identification, especially when combined with spectrofluorimetric analysis[15], but the specificity of the combination is generally still not high enough for unambiguous identification. Derivative analysis of GLC effluent[16,17] is highly specific

but requires extensive analytical equipment. Preparative TLC, together with the analysis of the isolated and purified material by a combination of physicochemical methods, is probably the most economic way to achieve the sensitivity and high specificity needed for competent toxicological work. Microcrystallography on material eluted from TLC plates has been proposed for the confirmation of drug identification[18].

In the present work the conditions have been investigated for the detection and identification of some central stimulants (compounds with stimulating action on the central nervous system) and of narcotic analgesics in urine. The procedure involves extraction by an organic solvent, two-dimensional TLC of the extract and subsequent studies on colour development after chromophoric spraying of the TLC plate. The fluorescence emission, UV-absorption, GLC properties and methyl-orange reaction of the compounds are studied after recovery of the materials from the TLC plates. The system has been adapted for the identification of other classes of basic drugs in urine, *i.e.* sedatives, tranquillizers, tricyclic antidepressants, antihistamines and muscle relaxants having central action.

MATERIALS AND METHODS

Pharmaceutical materials

The compounds investigated are listed in Table I, where the generic as well as the rational names are given.

Thin-layer chromatography

Silica gel F_{254} aluminium sheets (20 × 20 cm) (E. Merck, Darmstadt, G.F.R.), layer thickness 0.25 mm, were used. No pre-conditioning of the plates was carried out prior to the chromatography. By means of a Hamilton syringe, 50 μg of the compound in ethanolic solution (1 mg/ml) were applied to the TLC plate at a spot situated 2 cm from the base of the plate and 2 cm from the right-hand margin. In order to accelerate the evaporation of the solvent, the plate was simultaneously exposed to a stream of hot air.

Desaga glass tanks with ground tops and glass lids were used for developing tanks. The inside surface of the tank was covered with filter-paper. The first developing solvent was freshly prepared methanol–12 *N* ammonia (49:1) (ref. 19). After development for 18 cm, the chromatogram was removed from the tank and the solvent was evaporated in a stream of hot air. The TLC plate was then placed in a second tank containing chloroform saturated with ammonia, and developed for 18 cm at right angles to the first run. The chromatogram was allowed to dry and viewed under UV light from a hand lamp (Mineralight, short-wave UV filter, Model SL 2537, UV Products Inc., South Pasadena, Calif., U.S.A.). Regions differing in colour from the background were marked out with a pencil.

Finally, the following sequence of spray reagents was applied to the chromatogram:

(1) 20 ml of ninhydrin reagent, freshly prepared (0.5% w/v reagent grade ninhydrin in acetone; E. Merck), from a distance of 15 cm (ref. 20), followed by exposure to UV light (Minuvis; Desaga, Heidelberg, G.F.R.), at 254 and 366 nm, for 3 min from a distance of 7 cm.

TABLE I

THE PHARMACEUTICAL COMPOUNDS STUDIED

Compound	*Generic name*	*Salt*
(a) Alkaloids		
4,5-Epoxy-3-hydroxy-17-methyl-6-oxo-morphinan	hydromorphone	hydrochloride
4,5-Epoxy-3-methoxy-17-methyl-6-oxo-morphinan	hydrocodone	ditartrate dihydrate
(−)-4,5-Epoxy-14-hydroxy-3-methoxy-17-methyl-6-oxo-morphinan	oxycodone	hydrochloride
(−)-4,5-Epoxy-3-ethoxy-6-hydroxy-17-methyl-morphinen	ethylmorphine	hydrochloride dihydrate
(−)-4,5-Epoxy-6-hydroxy-3-methoxy-17-methyl-morphinen	codeine	phosphate sesquihydrate
(−)-4,5-Epoxy-3,6-dihydroxy-17-methyl-7-morphinen	morphine	hydrochloride trihydrate
4,5-Epoxy-6-hydroxy-17-methyl-3-(2-morpholinoethoxy)-morphinen	pholcodine	(base)
1,2,3,4,5,6-Hexahydro-6,11-dimethyl-3(3-methyl-2-butenyl)-2,6-methano-3-benzazocin-8-ol	pentazocine	(base)
Ethyl-1-methyl-4-phenyl-4-piperidinecarboxylate	pethidine	hydrochloride
4-(3-Hydroxyphenyl)-1-methyl-4-propionyl-piperidine	ketobemidone	hydrochloride
Ethyl-1-(3-hydroxy-3-phenylpropyl)-4-phenyl-4-piperidine carboxylate	phenoperidine	hydrochloride
N-(1-Phenethyl-4-piperidyl)propionanilide	phentanyl	citrate
(+)-1-(3-Methyl-4-morpholino-2,2-diphenyl-butyryl)pyrrolidine	dextromoramide	ditartrate
(+)-3-Methyl-4-dimethylamino-1,2-diphenyl-2-propionylpiperidine	dextropropoxyphene	hydrochloride
(±)-6-Dimethylamino-4,4-diphenyl-3-heptanone	methadone	hydrochloride
1-(3-Cyano-3,3-diphenylpropyl)-4-carbamoyl-piperidine	piritramide	tartrate
17-Allyl-4,5-epoxy-3,6-dihydroxy-7-morphinen	nalorphine	hydrochloride
1-Methyl-2-(3-pyridyl)pyrrolidine	nicotine	(base)
1,2,3,6-Tetrahydro-1,3,7-trimethyl-2,6-dioxo-purine	caffeine	(base)
8-[Hydroxy(6-methoxy-4-quinolinyl)methyl]-3-vinyl quinuclidine	quinine	(base)
(b) Central stimulants		
(±)-2-Amino-1-phenylpropane	amphetamine	sulphate
2-Diethylamino-1-phenyl-1-propanone	amphepramon	hydrochloride
2-(4-Chlorophenyl)-1,1-dimethylethylamine	chlorphentermine	hydrochloride
(+)-2-Amino-1-phenylpropane	dexamphetamine	(base)
2-Phenylethylammonium chloride	β-phenylethylamine	hydrochloride
2-Phenyl-1,1-dimethylamine	phentermine	hydrochloride
(+)-1-Phenyl-2-methylaminopropane	metamphetamine	hydrochloride
Methyl-2-phenyl-2-(2-piperidyl)acetate	methyl phenidate	hydrochloride
3-Methyl-2-phenylmorpholine	phenmetrazine	hydrochloride

(2) Sulphuric acid reagent (concentrated sulphuric acid–ethanol–water, 2:2:1) until moisture was visible on the surface of the plate, followed 5 min in an oven at 100° (ref. 21).

(3) Iodoplatinate reagent [To 1 volume of 10% w/v aqueous hexachloroplatinate(IV) (purum, *ca.* 40% Pt., Kebo AB, Stockholm, Sweden) were added 25 volumes of 4% w/v aqueous potassium iodide, after which water was added to give a final concentration of hexachloroplatinate(IV) of 0.1% w/v] applied until moisture was visible on the plate, followed by drying in a stream of hot air[22].

(4) Dragendorff reagent. Stock solution A: 2.1 g of basic bismuth(III) nitrate (reagent grade, E. Merck) were dissolved in 100 ml of distilled water; 25 ml of concentrated acetic acid were then added. Stock solution B: 50 g of potassium iodide were dissolved in 125 ml of distilled water. Spray reagent: stock solution A–stock solution B–concentrated acetic acid–water (1:1:2:10).

After each step the plate was inspected with regard to colour development. Finally the chromatograms were enclosed in parafilm and kept for future inspections.

Gas–liquid chromatography

Varaport 30–3% SE 30. Silicone SE 30 (Applied Science Labs., State College, Pa., U.S.A.) was dissolved in chloroform, and Varaport 30 (80/11 mesh; Varian Aerograph, Walnut Creek, Calif., U.S.A.) was added to give a final concentration of SE 30 of 3% w/v. The solvent was evaporated in a rotating vacuum flask under hot water. The packing material was added to a U-shaped glass column (6 ft. × 26 mm I.D.) by application of gentle suction. The column was mounted in an F & M Biomedical Model 400 gas chromatograph supplied with a flame ionization detector, and connected to a Honeywell writer (paper speed, 0.63 cm/min). Injection temperature, 320°; column temperature, 210°; hydrogen flow-rate, 40 ml/min; nitrogen flow-rate, 35 ml/min; air flow-rate, 600 ml/min.

Chromosorb G–5% Carbowax 20M–5% KOH. Equal amounts of Carbowax 20M (Hewlett-Packard, Böblingen, G.F.R.) and potassium hydroxide were separately dissolved in absolute ethanol, and the solutions were then combined. Chromosorb G was added to give a final concentration of 90% w/w dissolved constituents. After evaporation of the solvent the packing material was added to a spiral glass column (6 ft. × 2.8 mm I.D.), as described above. The column was mounted on a Perkin-Elmer F 11 gas chromatograph with flame ionization detector, and connected to a Hitachi–Perkin-Elmer 159 writer (paper speed, 1 cm/min). Injection temperature, 225°; column temperature, 190°; hydrogen flow-rate, 40 ml/min; nitrogen flow-rate, 35 ml/min; air flow-rate, 600 ml/min.

Chromosorb G–2.5% SE 30. SE 30 was dissolved in chloroform and Chromosorb G (80–100 mesh; Hewlett-Packard) was added to give a final concentration of 97.5% w/w dissolved material. The solvent was evaporated and the packing material added to the column as described above. The column was of the same type as that used for Carbowax–KOH and was mounted in the same oven of a Perkin-Elmer F 11 chromatograph. The operating conditions were identical to those described for the Carbowax column.

Trimethylsilyl ether derivatives. The material was dissolved in 100 μl of pyridine. 20 μl hexamethyldisiloxane (HMDS; Hopkin & Williams, Chadwick Heath, Great Britain) and 10 μl of chlorotrimethylsilane (CTMS; Hopkin & Williams) were added and the mixture was kept at 50° in a water-bath for 30 min.

Methyl orange reaction

A modified form of the ion-pair extraction procedure described by Brodie *et al.*[19] and by Axelrod[20] was used. 4 ml of benzene (reagent grade; washed with 1/10 volume of 0.1 *N* sodium hydroxide, with 0.1 *N* hydrochloric acid and twice with deionized water), 0.2 ml of isopentanol (reagent grade, washed as described for benzene), 20 μl of concentrated sodium hydroxide and 0.3 ml of methyl orange reagent [made immediately prior to use by mixing equal volumes of aqueous methyl orange (0.5 g per 100 ml, washed five times with equal volumes of chloroform) and 0.2 *M* borate buffer (pH 10)] were added to the compound to be tested and the tube was shaken for 5 min. After centrifugation, 2.5 ml of the benzene phase were transferred to a glass tube, 0.5 ml of ethanolic sulphuric acid (absolute ethanol containing 2% v/v of concentrated H_2SO_4) were added and the absorbance at 525 nm was recorded within 1 min of the addition.

Spectrophotometry

UV spectra were recorded on a Unicam SP 800 A spectrophotometer. Fluorescence spectra were recorded on a Perkin-Elmer MPF 2 A fluorescence spectrophotometer. Absorbances in the visible region were determined in a Beckman B apparatus in 1-cm cells.

RESULTS

Extraction of alkaloids and central stimulants from aqueous solution by use of chloroform–isopropanol at various pH values

The pharmaceutical compounds in ethanolic solution (1 mg/ml) were added to 0.1 *M* aqueous monosodium phosphate to give a concentration of 4.8 mg/ml. Saturated aqueous sodium borate was added to give a concentration of 10% v/v. Five aliquot portions of the resulting solution were adjusted to pH 8.5, 9.5, 10.5, 11.5 and 12.5, respectively. Water was added to give a final concentration of the pharmacological compounds of 4.0 mg/ml. After checking the pH, four 6-ml aliquot portions of each of these five solutions were extracted with 6 ml of chloroform–isopropanol (9:1). The organic phase was recovered by centrifugation. 5 ml of the extract were evaporated in the presence of 20 μl of concentrated HCl, and the dry residue was redissolved in 100 μl of ethanol. 2 μl of the ethanolic solution were injected as the trimethylsilyl (TMS) derivative. Nicotine, caffeine and the central stimulants, with the exception of methyl phenidate, were analyzed on the Chromosorb G–Carbowax column. Methyl phenidate was injected into the Chromosorb G–SE 30 column.

In a second experiment the chloroform–isopropanol extraction was made twice, and the procedure then performed as described above.

In Figs. 1 and 2 are shown the results of extractions of alkaloids and central stimulants, respectively, at various pH values. The ranges of the results of the four individual analyses are indicated. Broken lines represent the results from the single-extraction procedure, solid lines the results from repeated extractions. It is seen that methyl phenidate, metamphetamine, hydromorphone, morphine, ketobemidone and nalorphine were poorly extracted from aqueous solution at pH $>$ 10. The remaining compounds were in general equally well extracted at pH 9 as under more alkaline

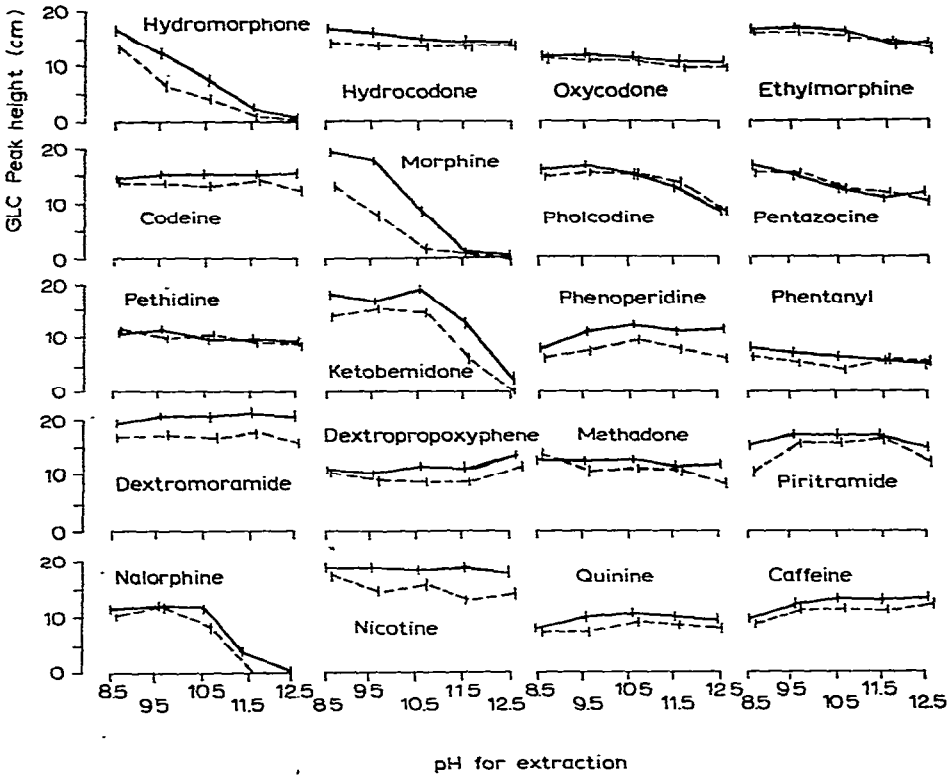

Fig. 1. Extraction of alkaloids from aqueous solution by chloroform–isopropanol (9:1) at various pH values.

conditions. Especially in the case of morphine, repeated extractions resulted in a considerably increased yield of material in the extract compared with a single extraction step.

Thin-layer chromatography

Two-dimensional TLC was performed as described in Materials and methods, 25 μg of substance being applied to the TLC plate in ethanolic solution (1 mg/ml). In Fig. 3 the results obtained on chromatography of the narcotic analgesics are shown, and in Fig. 4 those of the central stimulants.

In the amounts applied to the TLC plates, all of the compounds tested could be visualized under UV light. Amphetamine, dexamphetamine, metamphetamine, phenylethylamine and amphepramon gave violet spots after spraying with ninhydrin and UV exposition. The spots turned crimson after spraying with sulphuric acid, while iodoplatinate gave purple colours and the Dragendorff reagent gave various shades of orange. Only in the case of metamphetamine was the colour intensity increased on treatment with the iodoplatinate and Dragendorff spray reagents. Phenmetrazine, methyl phenidate, phentermine and chlorphentermine gave no coloured spots with ninhydrin or sulphuric acid. On spraying with iodoplatinate, methyl phenidate gave an orange spot, whereas the other central stimulants gave

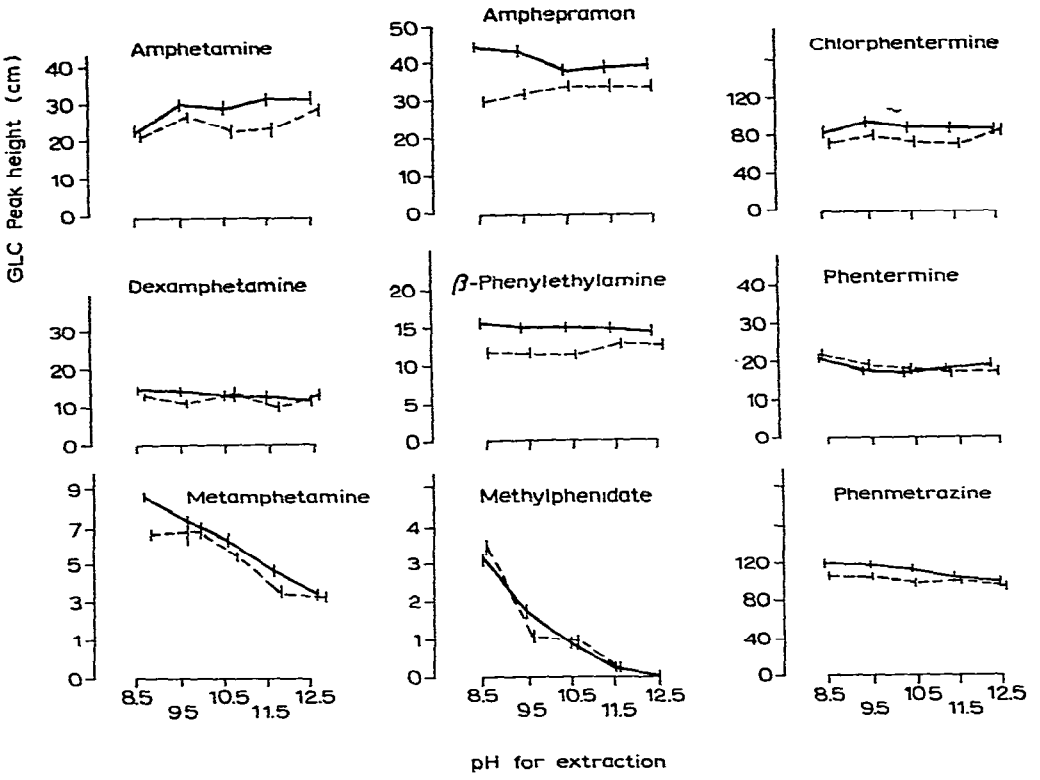

Fig. 2. Extraction of central stimulants from aqueous solution by chloroform–isopropanol (9:1) at various pH values.

purple spots. With the Dragendorff reagent the four compounds yielded orange spots of higher intensity than obtained with the iodoplatinate reagent. None of the narcotic analgesics gave coloured spots with ninhydrin or sulphuric acid. The spots produced by the iodoplatinate reagent varied in shades of purple between the different substances, but also varied with the concentration of the individual compound. Nicotine and caffeine differed from the rest of the substances tested in giving grey spots with iodoplatinate. With the Dragendorff reagent, morphine and nalorphine gave grey spots, whereas the other compounds gave spots having various shades of brown.

UV absorption spectra of alkaloids and central stimulants recovered by preparative TLC

The ethanolic solutions (1 mg/ml) of the pharmaceutical compounds were applied to the TLC plates and chromatographed in two dimensions as described in Material and methods. 500 μg were used of the central stimulants, hydromorphone, hydrocodone, oxycodone, ethylmorphine, codeine, morphine, pentazocine, ketobemidone, nalorphine, nicotine, quinine and caffeine, and 600 μg of pethidine, phenoperidine, phentanyl, dextromoramide, dextropropoxyphene, methadone and piritramide. The positions of the spots in the chromatograms were determined by inspection under UV light. Areas of silica gel containing the compounds under study were scraped off from the alumina foil support and transferred to glass tubes fitted

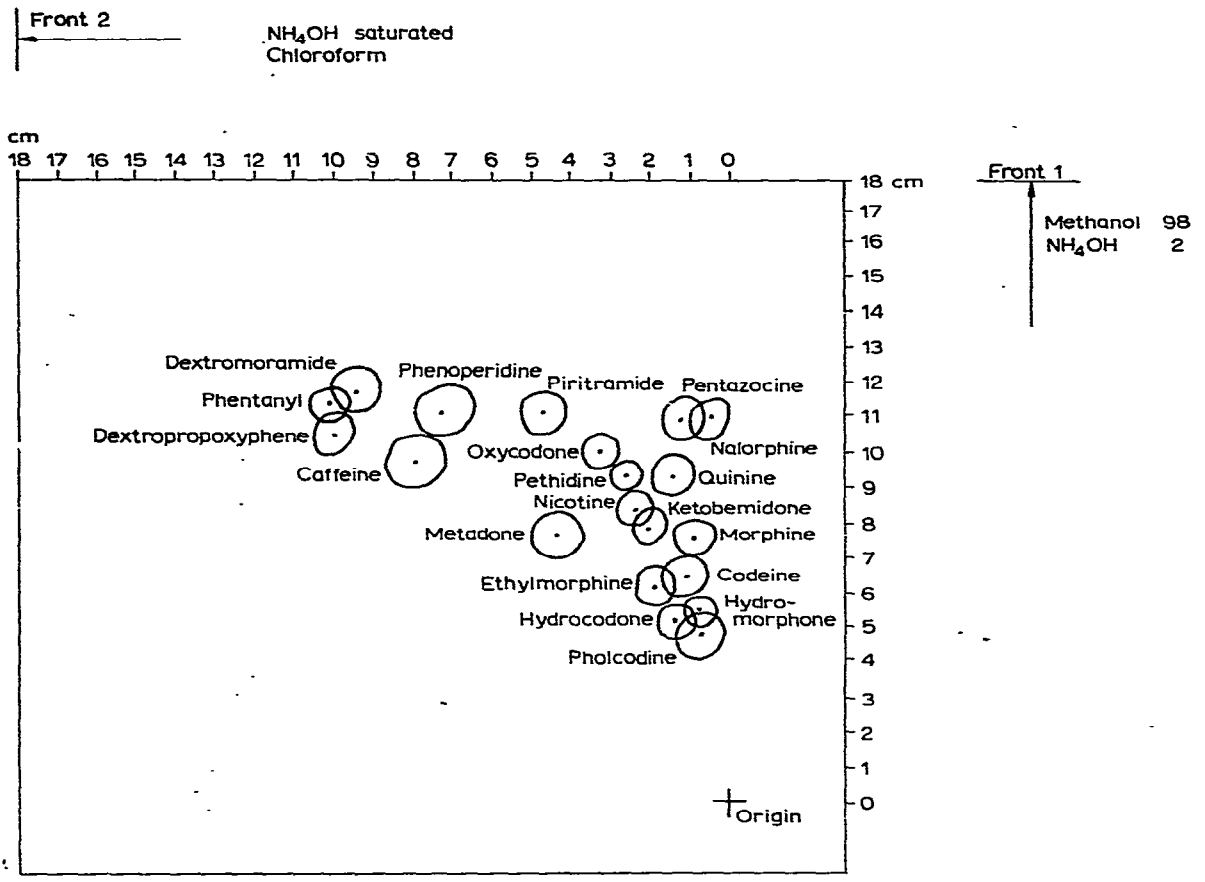

Fig. 3. Two-dimensional TLC of alkaloids on silica gel aluminium sheets. Solvents: 1 = methanol–ammonia (49:1); 2 = ammonia-saturated chloroform.

with ground-glass stoppers. After the addition of 1.5 ml of 0.1 *N* HCl and shaking for 5 min, the mixtures were centrifuged. An undiluted or suitably diluted (0.1 *N* HCl) aliquot portion of the hydrochloric acid phase was then transferred to a 1-cm quartz cuvette and the UV absorption spectrum was recorded. 40 μl of concentrated sodium hydroxide were added, and the spectrum was again recorded. The following dilutions of the extracts were made prior to the UV analysis: hydromorphone, 1:2; codeine, morphine and pentazocine, 1:2.5; ketobemidone, 1:4; nicotine, 1:5; quinine and caffeine, 1:10.

In one experiment a region of the developed TLC plate which was devoid of pharmaceutical substance was subjected to extraction with 0.1 *N* HCl. The UV absorption spectrum of the extract before (solid line) and after addition (broken line) of sodium hydroxide is shown in Fig. 5. In Fig. 6 the UV absorption spectra of amphepramon, amphetamine and chlorophentermine eluted from the TLC plate are represented by solid (acid solution) or broken bold lines (alkaline solution). The remaining central stimulants behaved like amphetamine and are not included in the

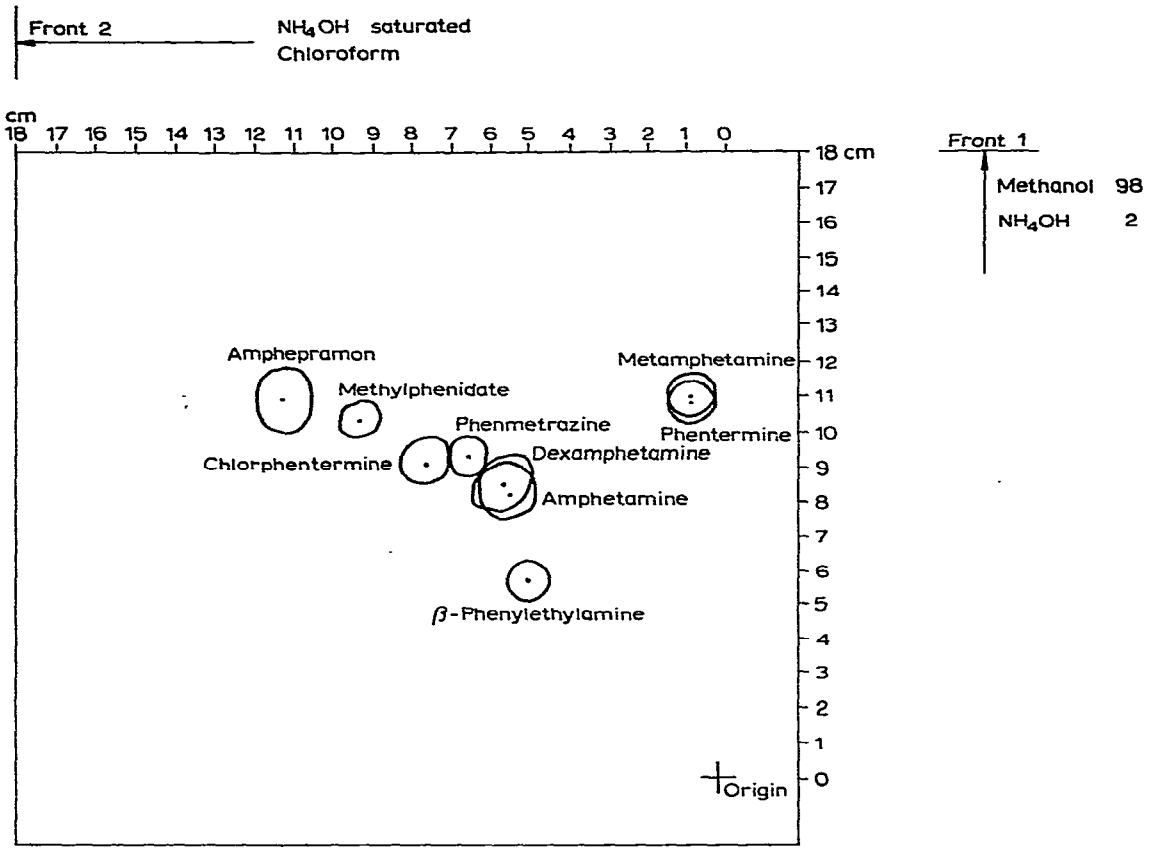

Fig. 4. Two-dimensional TLC of central stimulants. For details see Fig. 3.

figure. The normal solid and broken lines represent UV spectra, before and after addition of NaOH respectively, of ethanolic standards of the compounds added to 0.1 *N* HCl. In Fig. 7 the results obtained from TLC-processed alkaloids are shown as bold lines, together with the UV spectra obtained when ethanolic standards were added directly to HCl (normal lines). Where peak shifts occurred on adding NaOH, the spectra obtained are given as broken lines.

After TLC and recovery in 0.1 *N* HCl, all of the substances had kept their original UV absorbing spectra superimposed on a low background derived from the TLC material. In contrast, on addition of NaOH the background showed a drastic and unspecific increase which could not be nullified by centrifugation. The characteristic peak shifts exhibited by some of the compounds could, however, still be demonstrated. The UV absorption spectra obtained after TLC were thus found to be in close agreement with those of the unchromatographed materials and with those reported in the literature. With the exception of amphepramon, all of the central stimulants exhibited fine structure in the region 250–270 nm. In acid solution chlorphentermine gave an additional peak at 575 nm, shifting to 577 nm on addition of NaOH. Amphepramon had one main absorption maximum at 253 nm in acid

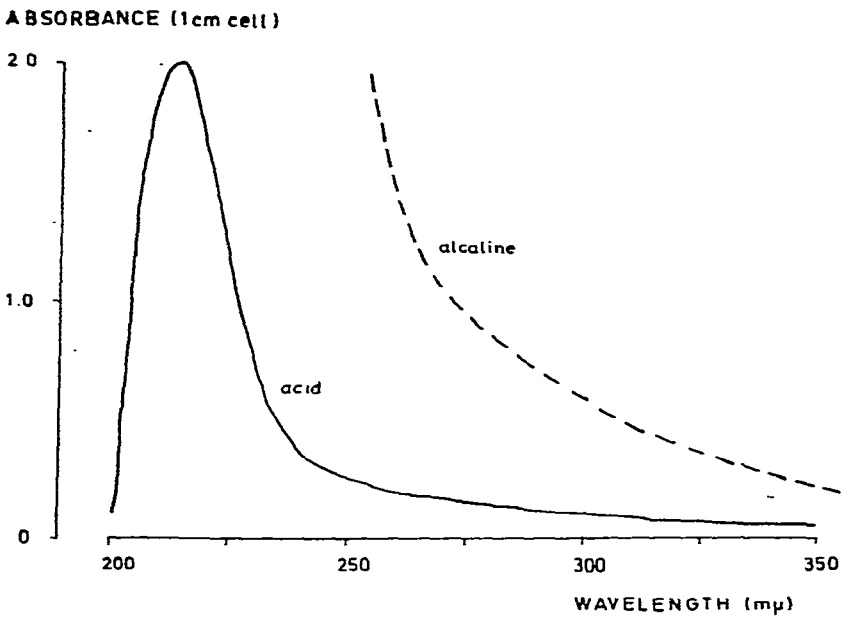

Fig. 5. UV spectrum of a hydrochloric acid extract of TLC plate material, before (solid line) and after (broken line) addition of sodium hydroxide.

solution, shifting to 248 nm in alkaline solution. The alkaloids fall into two categories with regard to the magnitude of their molar absorptions. Thus the iminoethanophenanthrofurans, pentazocine and ketobemidone, have 5–10 times higher molar absorptions than the remaining compounds. In contrast phentanyl, the piperidine

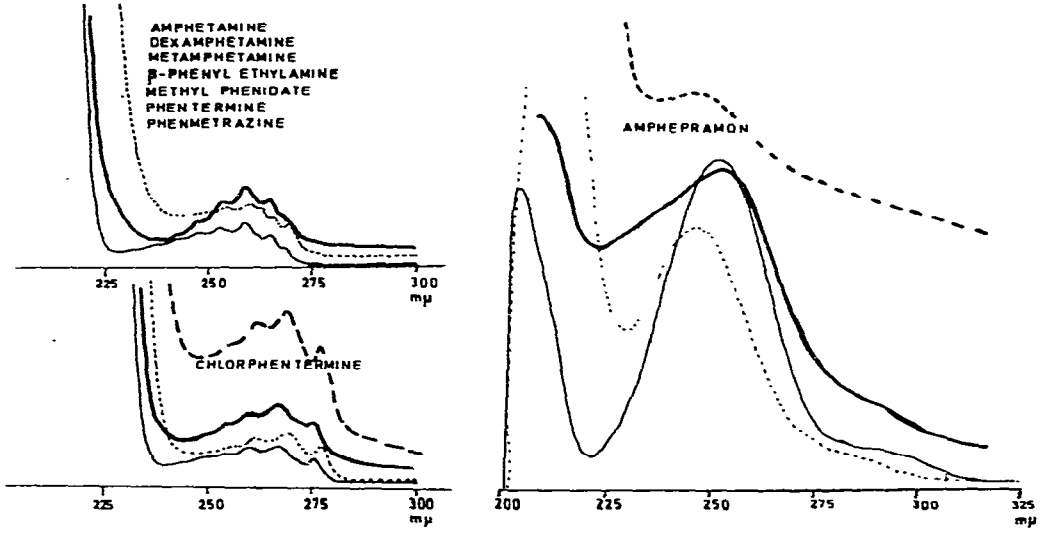

Fig. 6. UV spectra of amphepramon, amphetamine and chlorphentermine when added to 0.1 *N* hydrochloric acid (normal lines) and when recovered from processed TLC plates by extraction into HCl (bold lines). Broken lines represent the spectra obtained after addition of NaOH to the solutions.

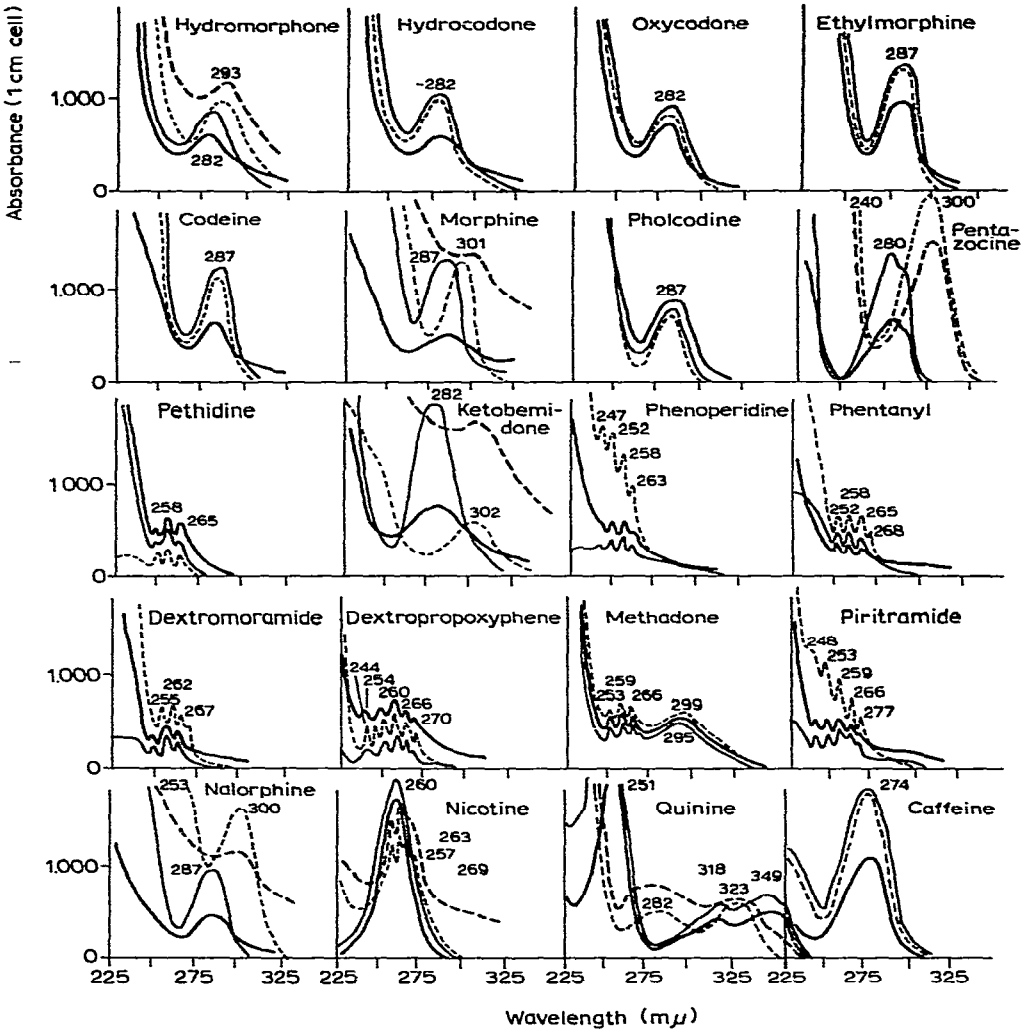

Fig. 7. UV spectra of some analgesic alkaloids, and of nalorphine, nicotine, quinine and caffeine, when added to 0.1 *N* HCl (normal lines) and when recovered from processed TLC plates by extraction into HCl (bold lines). The spectra obtained after addition of NaOH are represented by broken lines.

carboxylates of pethidine and phenoperidine and the four diphenylpropylamine derivatives exhibit low molar absorptions, the spectra being dominated by the benzene-type fine structure in the region 250–270 nm. The hydrochloride of methadone in addition has a peak at 295 nm which shifts to 299 nm in alkaline solution. Also pentazocine, ketobemidone, nalorphine, nicotine and quinine exhibit characteristic spectral changes when passing from acid to alkaline solution.

The recoveries of the substances after preparative TLC, as calculated from the UV absorptions of the hydrochloric acid extracts at the absorption maximum of the compounds, are given in Table II. The results of a recovery study based on GLC

TABLE II

RECOVERIES OF ALKALOIDS AND CENTRAL STIMULANTS

Recoveries after preparative TLC and (a) extraction into hydrochloric acid, as measured by UV absorption, and (b) extraction by hydrochloric acid and subsequent extraction into organic solvent as measured by GLC.

Alkaloid	*Recovery (%)*		*Central stimulant*	*Recovery (%)*	
	(a)	*(b)*		*(a)*	*(b)*
Hydromorphone	68	58	Amphetamine	56	54
Hydrocodone	78	75	Amphepramon	20	12
Oxycodone	76	80	Chlorphentermine	64	61
Ethylmorphine	75	75	Dexamphetamine	55	55
Codeine	79	81	Phenylethylamine	74	51
Morphine	73	61	Phentermine	78	42
Pholcodine	65	76	Metamphetamine	57	51
Pentazocine	52	41	Methyl phenidate	47	45
Pethidine	55	37	Phenmetrazine	78	49
Ketobemidone	82	74			
Phenoperidine	35	31			
Phentanyl	35	42			
Dextromoramide	41	30			
Dextropropoxyphene	44	42			
Methadone	55	38			
Piritramide	59	44			
Nalorphine	75	73			
Nicotine	32	29			
Quinine	59	12			
Caffeine	38	35			

(see below) are also given. The recoveries of substances in the hydrochloric acid extracts were generally in the 35–80% range. In the case of amphepramon, heavy trailing in the second solvent is responsible for the poor recovery.

Gas–liquid chromatography of ethanolic standards of alkaloids and central stimulants and of the same compounds recovered by preparative TLC

The GLC systems used and the operating conditions applied are described in Materials and methods. The compounds were dissolved in ethanol immediately prior to the analysis. 2 μl of the ethanolic solution, corresponding to 2 μg of substance, were injected into the Varaport 30–SE 30 and the Chromosorb G–Carbowax columns and the detector response was followed for 30 min. From the ethanolic solutions of the alkaloid compounds, 100 μl was removed and dried, redissolved in pyridine and treated with HMDS and CTMS. 2 μl of the resulting solutions, corresponding to 2 μg of substance, were injected into the Varaport 30–SE 30 column.

With the exception of methyl phenidate, all of the central stimulants, as well as caffeine and nicotine, were detected on the Chromosorb G–Carbowax column. None of the other alkaloids gave significant peaks in this system (Fig. 8). Methyl phenidate was well separated on the Chromosorb G–SE 30 column. All of the narcotic analgesics were detected in the Varaport 30–SE 30 system (Fig. 9); pholcodine and piritramide, however, were only detected after elevation of the column temperature. Quinine was detected only as the silyl derivative. None of the central

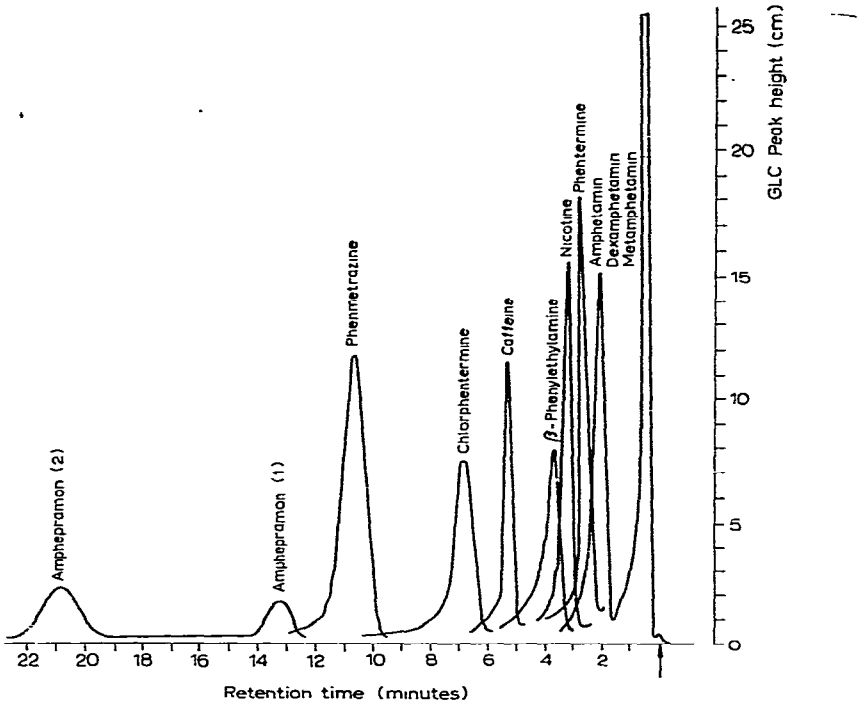

Fig. 8. GLC (on Chromosorb G–Carbowax) of central stimulants and of nicotine.

stimulants gave peaks on Varaport 30–SE 30. The TMS derivatives generally had longer retention times and yielded larger detector responses than the parent compounds. The use of repeated HMDS injections was found to improve greatly the sensitivity of the GLC system.

The pharmaceutical materials were then subjected to two-dimensional TLC as described in Materials and methods, 200 μg of substance being applied to the plate in ethanolic solution (1 mg/ml). By inspection under UV light, the compounds were localized on the chromatogram and the areas of silica gel were scraped off and transferred to glass tubes fitted with glass stoppers. 2 ml of 0.1 *N* HCl were added and, after shaking for 5 min, each mixture was centrifuged. 1 ml of a saturated aqueous solution of sodium tetraborate was added to a 1-ml aliquot portion of the eluate from the TLC plate and the mixture was adjusted to pH 8.5. 6 ml of chloroform–isopropanol (9:1) were then added. After shaking for 5 min, the organic phase was recovered by centrifugation. The remaining aqueous phase was adjusted to pH 9.5 and the extraction with 6 ml of chloroform–isopropanol was repeated. The extracts were combined and, after addition of 10 μl of concentrated HCl, they were evaporated to dryness at 60° under a stream of nitrogen. The dry materials were dissolved in 100 μl of ethanol. 2 ml of the ethanolic solution were then chromatographed in the GLC system best adapted for the detection of the individual compound, as established in the previous experiment.

All of the compounds tested could be detected and identified by their GLC retention times in the extracts from preparative TLC. The recoveries of the sub-

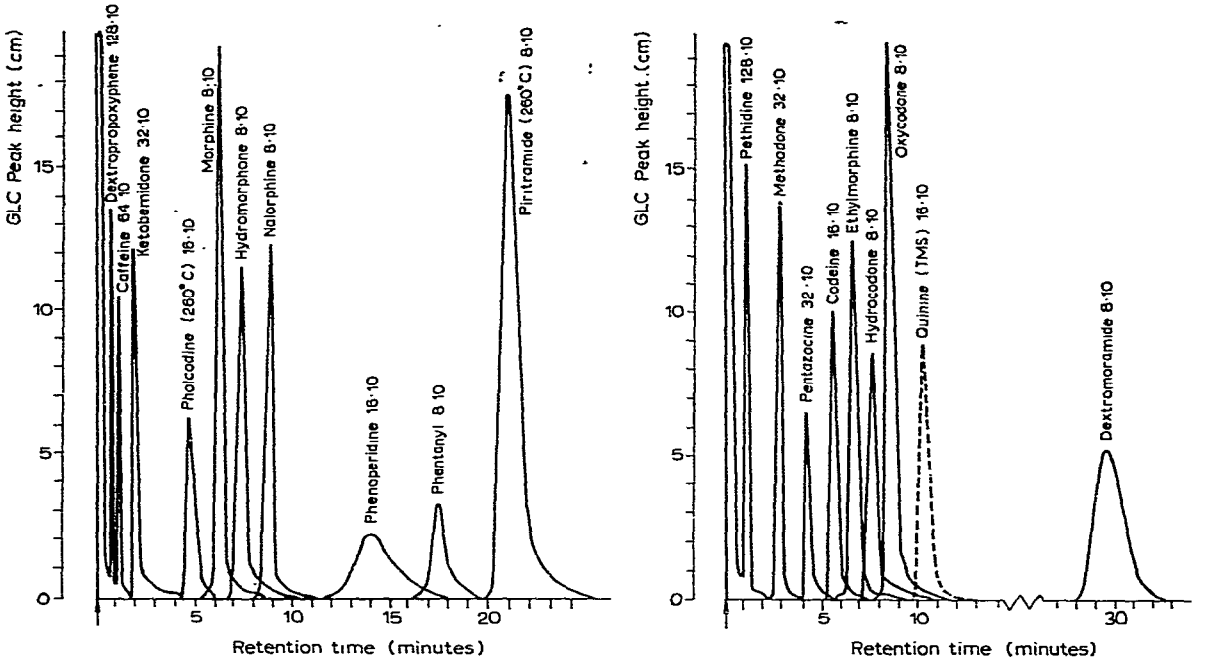

Fig. 9. GLC (on Varaport 30–SE 30) of some analgesic alkaloids and of nalorphine, quinine and caffeine.

stances, as calculated from the GLC peak areas after preparative TLC, subsequent extraction into HCl and further extraction into organic solvent, are given in Table II. It is noted that the recoveries were mainly dependent the path lengths of the substances in the two developing solvents.

Fluorescence spectra of alkaloids and central stimulants after TLC and chromophoric spraying

The compounds were chromatographed as described in Materials and methods, 100 μl of the ethanolic solution (1 mg/ml) being applied to the TLC plate. After visualization of the compound on the chromatogram by application of the sequence of spray reagents, the silica gel layer containing the substance was scraped off from the alumina foil support and transferred to a glass tube fitted with a ground-glass stopper. 2 ml of concentrated H_2SO_4 were added, and the stoppered tube was placed in a water-bath at 50° for 8 min. After the addition of 1 ml of distilled water, two pellets of sodium hydroxide and 2 ml of concentrated ammonium hydroxide, the stoppered tube was kept at 50° for 2 h. 3 ml of isobutanol were added and the tube was shaken for 5 min. The organic phase was recovered by centrifugation and used for fluorescence studies, 365 nm being the wavelength used for excitation[21].

In one experiment, regions of varying size from the developed and sprayed TLC plate which were devoid of pharmaceutical material were subjected to the procedure described above.

As demonstrated in Fig. 10, morphine, ethylmorphine, codeine, pholcodine

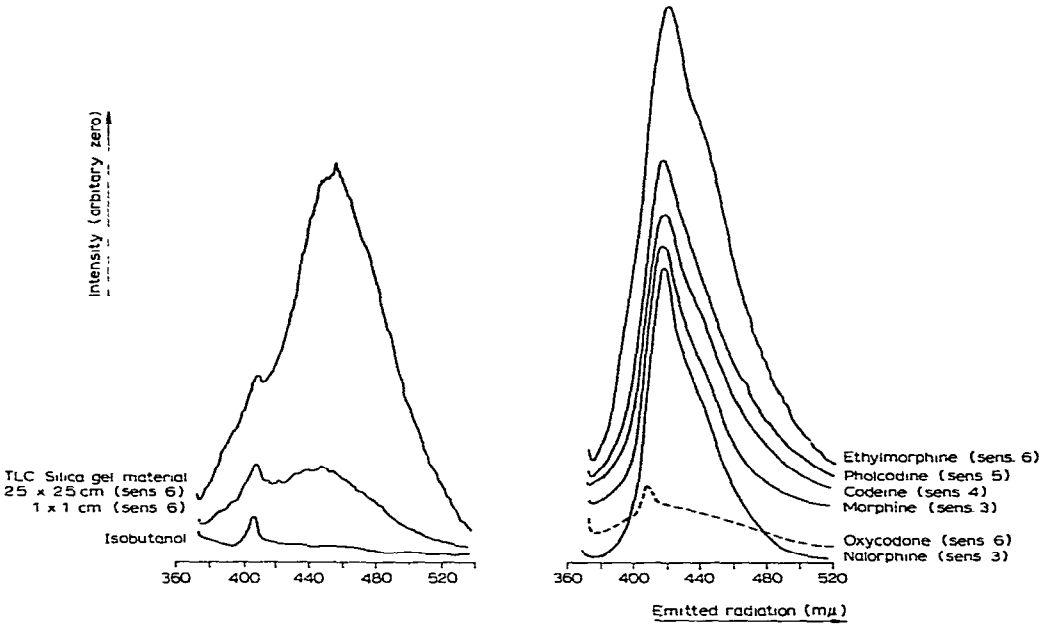

Fig. 10. Fluorescence emission spectra of processed TLC material and of ethylmorphine, pholcodine, codeine, morphine, oxycodone and nalorphine. The compounds were recovered from a TLC chromatogram which had been treated with a sequence of spray reagents.

and nalorphine gave distinct single-peak emission spectra, with maxima at 418 nm. The remaining compounds (*e.g.*, oxycodone) only gave small peaks at 410 nm and broad maxima at 440–450 nm, both of low intensity, derived from the processed TLC material (Fig. 10, TLC silica gel material).

Methyl-orange reaction of alkaloids and central stimulants recovered by preparative TLC

The availability of the compounds for ion-pair extraction with the methyl-orange reagents was investigated by use of ethanolic standards (1 mg/ml). Volumes (triplicate) corresponding to 10, 30 and 50 μg of the individual substances were evaporated (at 60° in a water-bath, with a stream of nitrogen) in the presence of 10 μl of glacial acetic acid. To the dry residues, 4 ml of benzene were added, and the analysis was performed as described in Materials and methods. In Figs. 11 and 12 the absorbances obtained are plotted against the amount of alkaloid and central stimulant, respectively, used for the analysis. The ranges and mean values of the absorbances of each compound are also indicated. With the exception of caffeine, all of the compounds tested showed a positive methyl-orange reaction. However, considerable variations in colour yield were noted for the different compounds. Within the concentration range studied, in each case the absorbance was directly related to the amount of substance tested.

The ethanolic standards were applied to the TLC plates in amounts corresponding to 100, 200 and 300 μg of each compound. Each amount was run on duplicate plates. Chromatography was performed in two dimensions as described in

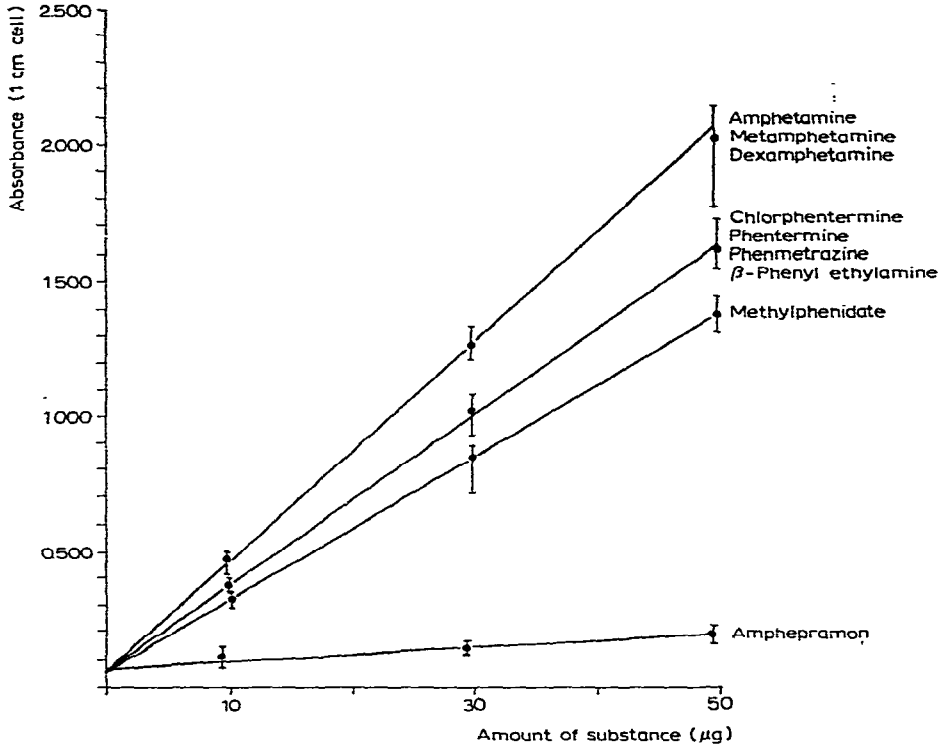

Fig. 11. Absorbances obtained in the methyl-orange reaction of some analgesic alkaloids and of nalorphine, nicotine, caffeine and quinine.

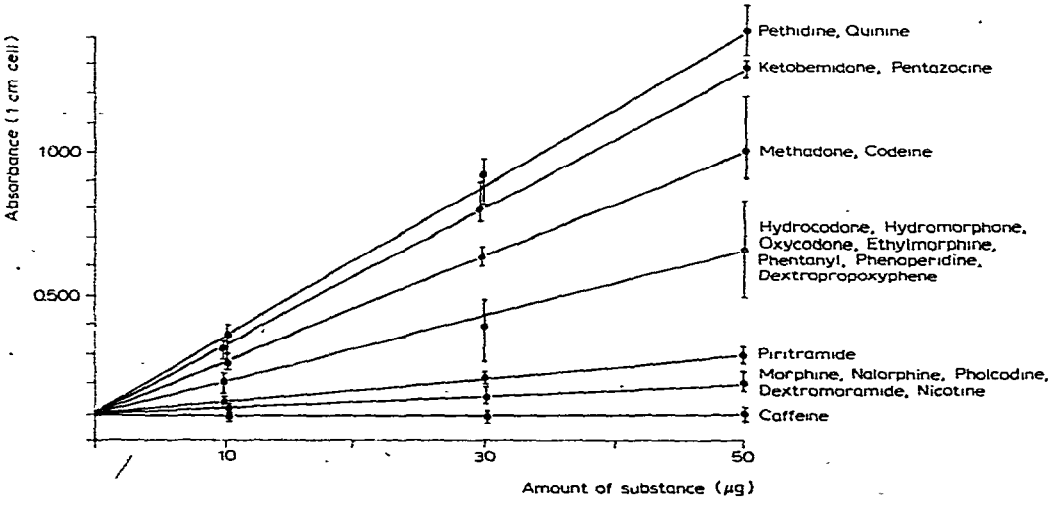

Fig. 12. Absorbances obtained in the methyl-orange reaction of central stimulants in various concentrations.

TABLE III

ABSORBANCES PRODUCED IN THE METHYL-ORANGE REACTION OF ALKALOIDS AND CENTRAL STIMULANTS ADDED TO TLC PLATES IN VARYING AMOUNTS AND RECOVERED AFTER TWO-DIMENSIONAL TLC

Alkaloids	*Absorbance from duplicate TLC runs*			*Central stimulants*	*Absorbance from duplicate TLC runs*		
	100 μg	*200 μg*	*300 μg*		*100 μg*	*200 μg*	*300 μg*
Hydromorphone	0.595	0.905	1.490	Amphetamine	0.320	0.645	1.090*
	0.675	1.290	1.075		0.295	0.740	0.905*
Hydrocodone	0.755	1.105	1.270	Amphepramon	0.110	0.136	0.210*
	0.760	0.985	1.470		0.170	0.145	0.190*
Oxycodone	0.395	0.565	0.935	Chlorphentermine	0.456	0.660	0.810*
	0.445	0.570	0.805		0.470	0.670	1.040*
Ethylmorphine	0.445	1.015	1.500	Dexamphetamine	0.330	0.715	1.110*
	0.575	1.195	1.390		0.190	0.770	0.730*
Codeine	0.280	0.605	0.810*	β-Phenylethylamine	0.125	0.105	0.085*
	0.195	0.510	0.870*		0.105	0.115	0.090*
Morphine	0.105	0.125	0.240	Phentermine	0.410	0.750	0.930*
	0.120	0.290	0.310		0.400	0.750	0.905*
Pholcodine	0.170	0.405	0.545	Metamphetamine	0.345	0.480	1.245*
	0.145	0.410	0.555		0.355	0.620	1.105*
Pentazocine	0.250	0.565	0.820*	Methyl phenidate	0.150	0.250	0.230*
	0.250	0.555	0.840*		0.090	0.155	0.255*
Pethidine	0.280	0.470	0.720*	Phenmetrazine	0.300	0.616	0.790*
	0.165	0.610	0.840*		0.190	0.530	0.950*
Ketobemidone	0.280	0.780	0.935*				
	0.320	0.790	0.870*				
Phenoperidine	0.500	1.040	1.370				
	0.555	0.940	1.250				
Phentanyl	0.325	0.785	1.205				
	0.255	0.930	1.065				
Dextromoramide	0.170	0.185	0.155				
	0.110	0.150	0.195				
Dextropropoxyphene	0.270	0.550	0.695				
	0.375	0.480	0.740				
Methadone	0.165	0.230	0.420*				
	0.160	0.200	0.380*				
Piritramide	0.166	0.830	1.460				
	0.445	0.745	1.220				
Nalorphine	0.160	0.285	0.600				
	0.185	0.370	0.465				
Nicotine	0.100	0.135	0.210				
	0.115	0.120	0.120				
Caffeine	0.125	0.125	0.085				
	0.095	0.120	0.090				
Quinine	0.295	0.550	0.815				
	0.325	0.530	0.765				

* The solution was diluted by a factor of 5.

Materials and methods. The positions of the compounds on the chromatograms were determined by inspection under UV light, and the pharmaceutical materials contained in the spots were recovered by the extraction procedures described in detail in the section on gas–liquid chromatography. After addition of 10 μl of glacial acetic acid, the final chloroform–isopropanol extract was taken to dryness at 60° under a stream of nitrogen. 4 ml of benzene were added to the dry residues, and the methyl-orange reaction was performed as described in Materials and methods. In the case of the central stimulants and the alkaloids of codeine, pentazocine, pethidine, ketobemidone, methadone and quinine, the benzene solutions of the compounds were diluted by a factor of 5 prior to the addition of the ethanolic sulphuric acid. The results of the duplicate runs of the different amounts of alkaloids and central stimulants, respectively, are given in Table III.

DISCUSSION

The results of the present investigation furnish the basis for a method for the detection and identification of basic psychoactive drugs in urine. The procedure involves extraction into an organic solvent and subsequent duplicate TLC of the extracted material. For localization and tentative identification of a compound, one of the chromatograms is treated with a sequence of chromophoric spray reagents. For further identification, material isolated from a visualized spot is used for fluorimetric studies. With the aid of the spots on the guide plate and by inspection under UV light, the compound is localized in the second chromatogram. The area of silica gel containing the substance is removed from the support and the compound is extracted into hydrochloric acid. The extract is then used for UV absorption studies under acid and alkaline conditions. The compound is then extracted into an organic solvent. After addition of one drop of acetic acid, the solvent is evaporated and the dry residue is dissolved in ethanol. A 2-ml aliquot portion of the resulting solution is used for GLC. The remaining solution is analyzed by the methyl-orange reaction. Detection thus depends on TLC and chromophoric spraying of the chromatogram. Identification rests on the parameters of extractability at the relevant pH, TLC R_F values in the two developing solvents, colour development on sequential application of the various spray reagents, fluorescence and UV absorption characteristics, GLC retention times in the different systems and on the results of ion-pair extraction with methyl orange.

The aim of the investigation was to develop a suitable procedure for the analysis of central stimulants as well as narcotic analgesics. Extraction by organic solvent was thus preferred to extraction by resin[13]. Chloroform was chosen rather than diethyl ether since the latter tends to extract larger amounts of urinary pigments which cause interference (*e.g.*, ref. 5). While hydromorphone, morphine, ketobemidone and methyl phenidate are practically inextractable at strongly alkaline pH, extraction at pH 8.5–9.5 results in good yields of all of the compounds tested. Thus for optimal sensitivity and applicability of the analytical procedure, duplicate chloroform–isopropanol extraction at pH 8.5 and 9.5 were chosen for transfer of the pharmaceutical compounds from the aqueous phase to the organic solvent.

Pre-coated silica gel TLC plates with an added fluorescence indicator can be used in the analytical procedure. This is of obvious practical implication. Pre-

conditioning of the plates is unnecessary, they are durable and easy to handle and are highly reproducible between the batches. After application of plastic film to the chromatograms, the plates can be easily preserved and filed for future inspection.

The methanol–ammonia used as the first developing solvent has been proposed[22] for the general screening of nitrogenous bases, and has also been employed by several workers for toxicologic TLC. The separation characteristics of this solvent make it well suited for use on extracts from urine, the majority of the alkaloids and stimulants being distributed at R_F values of less than 0.55, *i.e.*, below the region where the bulk of the interfering substances present in urine are found. "Steele's solvent"[23] was tested as a second developing solvent. Very satisfactory results were obtained with respect to the critical separations of the fast-moving compounds in the first solvent. However, because of the potential danger connected with the use of acetonitrile, Steele's solvent was abandoned in favour of chloroform saturated with ammonia, which showed almost equally good separations of the compounds which exhibited high R_F values in methanol–ammonia. Thus the combination of the two developing solvents of methanol–ammonia and chloroform saturated with ammonia gives a two-dimensional TLC system where the first solvent separates the morphine derivatives, methadone, ketobemidone and most of the stimulants from the faster-moving compounds and from interfering urinary constituents, and where development in the second solvent results in a dispersion of the compounds with high R_F values. In contrast to the remaining compounds tested, the TLC-processed morphinens produced well defined single-peak fluorescence emission spectra when subjected to the treatment proposed by Nadeau and Sobolewski[21]. The shoulder observed in the region 440–450 nm is accounted for by the emission produced by the silica gel material. The presence of this background spectrum restricts the amount of TLC material which can be included in the analysis, and the amount removed from the chromatogram for fluorescence analysis should not exceed an area of *ca.* 1 cm^2.

In acid solution the components of the hydrochloric acid extract from the TLC chromatogram do not interfere with UV-absorption studies of the compounds contained in the silica gel layer. On addition of NaOH, however, an increase in optical density takes place, which is not reduced by centrifugation. In cases where larger amounts of TLC material are used in the extraction, this turbidity effectively blocks studies of UV absorption of the pharmaceutical material under alkaline conditions. When the area of the chromatogram removed for extraction is of the order of 1 cm^2 or less, the interference is not critical. The GLC systems used in the present investigation have previously been applied in toxicological work (*e.g.*, ref. 16). None of the systems react significantly with compounds occurring naturally in urine, as extracted under the conditions applied. The Varaport 30–SE 30 column, however, did produce a series of peaks when the injected solution was derived from a chloroform extraction performed in tubes having screw caps fitted with polythene packing. For this reason, tubes with ground-glass stoppers were used for the chloroform extractions.

Most of the compounds which yield a positive methyl-orange reaction, as investigated with the aid of standards added to benzene, could be recognized in extracts from TLC plates. In a few cases the response was very low or absent (*i.e.*, nicotine, phenylethylamine and dextromoramide), but generally the absorbance yielded by the TLC extract was related to the amount of compound added to the

TLC plate and to the TLC path length. The dispersion of the individual values of the duplicate analyses was rather large, and the usefulness of the procedure for quantitative work is correspondingly restricted. Nevertheless the applicability of the methyl-orange reaction to extracts from TLC plates for identification purposes is convincingly demonstrated.

The analytical procedure described has been thoroughly tested in routine toxicological work. It has also been used in studies of the drug habits of homeless men[24], of patients applying to a casualty ward[25,26] and of individuals receiving acute psychiatric care[27]. Applied to urine specimens treated with β-hyaluronidase, the system has shown to react to, and to be able to identify, the metabolic products of a large variety of basic psychotropic compounds, therapeutic as well as narcotic.

REFERENCES

1 V. P. Dole, W. K. Kim and I. Eglitis, *J. Amer. Med. Ass.*, 198 (1966) 349.
2 K. D. Parker and C. H. Hine, *Bull. Narcotics*, XIX (1967) 51.
3 B. Davidow, N. L. Petri and B. Quame, *Amer. J. Clin. Pathol.*, 50 (1968) 714.
4 V. Marsks and D. Fry, *Proc. Ass. Biochem.*, 5 (1968) 95.
5 D. J. Berry, J. Grove, B. Widdop and J. H. P. Willis, *Bull. Narcotics*, XXII (1970) 31.
6 N. Weissman, M. L. Lowe, J. M. Beattie and J. A. Demetriou, *Clin. Chem.*, 17 (1971) 875.
7 J. K. Brown, R. H. Schingler, M. G. Chaubal and M. H. Malone, *J. Chromatogr.*, 87 (1973) 211.
8 W. J. Woodford, *J. Chromatogr.*, 115 (1975) 678.
9 L. Lepri, P. G. Desideri and M. Lepori, *J. Chromatogr.*, 116 (1976) 131.
10 I. Sunshine, *J. Chromatogr.*, 82 (1973) 125.
11 I. Sunshine, W. W. Fike and H. Landesman, *J. Forensic. Sci.*, 11 (1966) 428.
12 I. Zingales, *J. Chromatogr.*, 31 (1967) 405.
13 S. J. Mulé, *J. Chromatogr.*, 39 (1969) 302.
14 M. L. Bastos, G. E. Kananen, R. M. Young, J. R. Monforte and I. Sunshine, *Clin. Chem.*, 16 (1970) 931.
15 S. J. Mulé, *J. Chromatogr.*, 55 (1971) 255.
16 A. H. Beckett, G. T. Tucker and A. C. Moffat, *J. Pharm. Pharmacol.*, 19 (1967) 273.
17 F. W. Karasek, H. H. Hill, Jr. and S. H. Kim, *J. Chromatogr.*, 117 (1976) 327.
18 J. R. Broich, D. B. Hoffman, S. Andryauskas, L. Galante and C. J. Umberger, *J. Chromatogr.*, 60 (1971) 95.
19 B. B. Brodie, S. Udenfriend and W. Dill, *Biol. Chem.*, 168 (1947) 319.
20 J. Axelrod, *J. Pharm. Exp. Ther.*, 110 (1954) 315.
21 G. Nadeau and G. Sobolewski, *Can. J. Biochem.*, 36 (1958) 625.
22 I. Sunshine, *Amer. J. Clin. Pathol.*, 40 (1963) 576.
23 J. A. Steele, *J. Chromatogr.*, 19 (1965) 300.
24 S. Thunell, *Toxicological Analysis*, in S. Borg, *Homeless Men. A social-psychiatric and Clinical Investigation Dissertation*, Karolinska Institute, Stockholm, 1974, p. 91.
25 S. Borg, S. Thunell and A. Åkerman, *Nord. Psykiat. Tidsskr.*, 30 (1976) 113.
26 S. Borg, S. Thunell, A. Åkerman and C. Allgulander, *Nord. Psykiat. Tidsskr.*, 2 (1977) 201.
27 S. Borg and S. Thunell, *Nord. Psykiat. Tidsskr.*, in press.