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AUTOTRANSFER CHROMATOGRAPHY COMBINED WITH MASS SPECTROSCOPY, FOR THE CHARACTERIZATION OF PYRROLES AND INDOLES

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SUMMARY

The need for a means of unambiguously identifying micro-amounts of metabolites in biochemical psychiatry provided the stimulus for linking autotransfer chromatography with high-resolution mass spectroscopy. The present procedure involves the sequence thin-layer chromatography, autotransfer, paper chromatography, elution, and mass spectrometry. This system has allowed the identification of "mauve factor" as kryptopyrrole. Supplementary information supporting this identification is also presented.

Broader applicability of combined autotransfer chromatography and high-resolution mass spectroscopy is suggested by the rapid, precise mass spectral identification of indole-3-acetic acid in crude urine extracts, without any modification of the procedure.

INTRODUCTION

In a previous paper[1] the North Battleford group gave a general definition of autotransfer chromatography (ATC) as chromatography in two or more dimensions, where the stationary phase is changed, and with facile, total transfer of sample components. This procedure, therefore, belongs to the class "transfer techniques" as defined by STAHL[2] at the Third Liblice Conference, and is related to the method of JANÁK, in which GLC effluent streams are automatically transferred to thin-layer plates or paper strips[3]. The subtype of ATC being considered here, namely transfer from TLC to paper partition chromatography, has been described in detail[1,4]. This subtype of ATC is extremely flexible. The thin layer and its developing solvent can both be changed, the transferring solvent can be changed, and the final developing solvent for the paper chromatographic step can be changed. Additionally, the paper can be loaded or pre-treated. Nevertheless, the present paper deals with the exact stationary, transfer and mobile phases described previously[1,4]. The purpose of this communication is to demonstrate the application of ATC, and ATC linked with high-

resolution mass spectrometry, in the identification of micro-amounts of pyrroles and indoles in complex mixtures.

Primary consideration is given to the identification of "mauve factor", an abnormal metabolite with promising clinical correlates in psychiatry[5,6]. The positive identification of indole-3-acetic acid in crude urine extracts is also presented, and demonstration of the molecule ion peak of 5-hydroxyindole-3-acetic acid in its presumptive ATC spot is recorded.

The clinical and historical aspects of this work need not be emphasized; suffice it to say that "mauve factor" is reliably associated with psychotic mental disorders[4–12] and that its chemical structure could not be identified for ten years. Although several laboratories were working on the problem and our early prediction[1] of a pyrrolic structure gained some support from the work of SOHLER *et al.*[7] with model substances, little real progress was made until ATC was applied, and confirmation of its molecular structure depended upon combining ATC with high-resolution mass spectrometry. Laboratory results bearing on the identification of this material are summarized in Table I.

In the work on "mauve factor", our first application of the enhanced resolution of ATC was to assess homogeneity; the second application was to rule out selected compounds which were isographic with the factor in other systems; the third was to direct our search to compounds isographic (and presumably identical) with "mauve factor" in the ATC system; and the fourth application was in the preparation of samples for high-resolution mass spectroscopy.

During the course of this work, it became quite apparent that "mauve factor" was highly labile and perplexingly inconsistent in some of its behavior. ATC and high-resolution mass spectrometry have also proved indispensible in clarifying some of the interesting side issues and additional chemical problems raised by this instability.

AUTOTRANSFER CHROMATOGRAPHY (TL → PA)

In earlier work with unidimensional paper chromatographic systems there had been some indication that "mauve factor" was heterogeneous[1,7,12]. When our standardized ATC (TL → Pa) was applied to the factor, it yielded a characteristic pattern (fingerprint) of five spots (Fig. 1). Next, ATC was used to eliminate from further consideration a number of compounds which were isographic with "mauve factor"

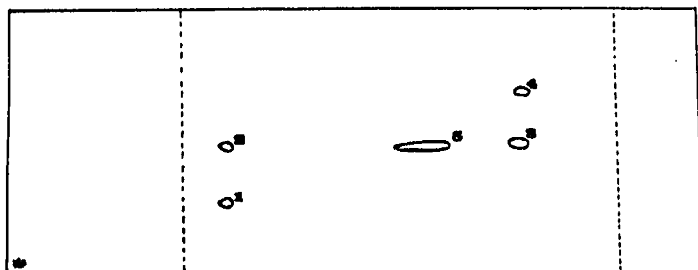

Fig. 1. Autotransfer chromatogram pattern of components attributable to kryptopyrrole ("mauve factor"). The star represents the position adjacent to the spot at application; the dotted lines represent the transfer solvent front and the paper chromatography solvent front.

TABLE I

COMPARISON OF "MAUVE FACTOR" AND KRYPTOPYRROLE SOLUTIONS

No.	*Procedure*	*Results*	
		"Mauve factor"	*Kryptopyrrole*
1	Paper electrophoresis (1/200 NH_4OH, 820 V)	Same position as urea	Same position as urea
2	Electronic transition spectroscopy		
	(a) Ehrlich's product visible range	Max. 525–535 nm Shoulder 565 nm Shoulder 615 nm	Max. 525–535 nm Shoulder 565 nm Shoulder 615 nm
	(b) Direct sample UV range	No peaks 218–340 nm	No peaks 218–340 nm
	(c) Fluorescence	Negative	Negative (but isomeric haemopyrrole fluoresces)
3	Chromogenic agents		
	(a) Initial battery[1] of aromatic aldehydes	Pattern matched only α-monosubstituted pyrroles	(Kryptopyrrole is an α-monosubstituted pyrrole)
	(b) Current results with "component 1"		
	(i) Ehrlich's reagent	Rose-purple	Rose-purple
	(ii) *p*-Dimethylamino-cinnamaldehyde	Pale rose	Pale rose
	(iii) Vanillin	Pale ochre	Pale ochre
	(iv) Benzaldehyde	Nil	Nil
	(v) Cinnamaldehyde	Pale cream	Pale cream
	(vi) *p*-Hydroxy-benzaldehyde	Rose-gold	Rose-gold
	(vii) 2,4-Dihydroxy-benzaldehyde	Rose-peach	Rose-peach
	(viii) Naphthaldehyde	Nil	Nil
	(ix) *o*-Phthalaldehydic acid	Nil	Nil
	(x) Xanthydrol 5%	Salmon	Salmon
	(xi) Ninhydrin followed by acid	Slowly gold	Slowly gold
	(xii) Isatin plus acid	Grey-blue	Grey-blue
4	Chromatography		
	(a) Column		
	(i) Ion exchange[1]	In neutral fraction	In neutral fraction
	(ii) Charcoal plus Fullers' earth	First substance to break through (highly selective)	Break-through point the same
	(b) Paper		
	(i) "IPrAm"[1]	R_F 0.88; sometimes second spot R_F 0.92	R_F 0.88; sometimes second spot R_F 0.94
		co-chromatography showed isography	
	(ii) "DIPREAM"[1]	R_F 0.88	R_F 0.88
		co-chromatography showed isography	
	(iii) "Sohler 8% NaCl"[7]	R_F 0.88	R_F 0.86
		co-chromatography showed isography	
	(iv) "Reio System D" M_1	R_F 0.08	R_F 0.08
	(M_1, M_2 etc. are component spots) M_2	R_F 0.08	R_F 0.08
	M_3	R_F 0.78	R_F 0.78
	M_4	R_F 0.85	R_F 0.84
	M_5	R_F 0.60, streak	R_F 0.60, streak

No.	Procedure		"Mauve factor"	Kryptopyrrole
	(v) "BA 50"	M_1	0.54	0.54
		M_3	0.88	0.91
	(c) Thin-layer (silicic acid/diethyl ether)		Three components:	Three components:
		M_1	R_F 0.24	R_F 0.24
		M_2, M_3, M_5	R_F 0.42	R_F 0.44
		M_4	R_F 0.56	R_F 0.58
	(d) Autotransfer[1,5] routine method, two-dimensional		Five components: (TLC, PC)	Five components: (TLC, PC)
		M_1	R_F (0.24, 0.08)	R_F (0.25, 0.08)
		M_2	R_F (0.42, 0.08)	R_F (0.44, 0.08)
		M_3	R_F (0.42, 0.78)	R_F (0.44, 0.78)
		M_4	R_F (0.56, 0.85)	R_F (0.58, 0.84)
		M_5	R_F (0.42, 0.60, streak)	R_F (0.44, 0.60, streak)
			All components isographic on	*co-autotransfer chromatography*
5	High-resolution mass spectroscopy			
	(A)		By three different methods of extraction characteristic peak at 123.106	On opening ampoule characteristic peak at 123.106
	(B)		Component 1[a]: N-containing peaks at: 260, 245, 137 and 123.	In water solution[a]: N-containing peaks at: 260, 245, 137 and 123.

[a] These high-resolution mass spectra are identical.

in the well-known isopropanol–ammonia–water unidimensional system. It was easily shown that "mauve factor" was not bufotenin and was not identical to any of over 60 available indolic substances. We paid particular attention to all the indoles which were in vogue in biochemical psychiatry, including the isomeric hydroxyskatoles, the psychotomimetic tryptamines, indole-3-acetamide, and the pineal substances. All of these were clearly different from the compounds yielding the characteristic mauve spot pattern.

With the discovery that resorcinol-type phenols and also mesityl oxide type ketones[13] yield indole-like colors with Ehrlich's reagent these were screened by ATC and likewise ruled out from further consideration. The model substance for "mauve factor", of SOHLER *et al.*, produced from hexosamines[7], was similarly shown to differ from "mauve factor" on ATC. But because of continuing indications that "mauve factor" was pyrrolic we decided to inject rats intraperitoneally with various pyrroles, diluting the urine and then applying our routine method for extracting and monitoring the "mauve factor". This work also proved largely negative, except that 2,4-dimethylpyrrole and 2,4-dimethyl-3-acetylpyrrole yielded an autotransfer "fingerprint" of Ehrlich spots similar to, although offset from, the pattern of "mauve factor" components. An analysis of the structure–R_F relationships suggested that a 2,4-dialkylpyrrole with perhaps an ethyl group in position 3 might yield the mauve factor pattern. This prediction was verified when kryptopyrrole, injected into rats, yielded the precise fingerprint.

The excretion products of the rats from kryptopyrrole were labile and rather inconsistent in the same ways that "mauve factor" had been in the past, and chro-

matographic studies on kryptopyrrole itself showed marked complexity and sensitivity to very mild conditions. But parallel prior treatment of "mauve factor" and kryptopyrrole yielded precisely parallel results. Co-ATC of "mauve factor" and kryptopyrrole showed isography of all respective components, while addition of kryptopyrrole to "mauve factor"-negative urine yielded a typical "mauve factor"-positive ATC, and processing an aqueous solution of kryptopyrrole in place of urine again yielded the identical pattern. At this point, a basic relationship had been established between kryptopyrrole and "mauve factor" and the tentative identification had been made largely on the basis of the unique properties of this material in our standard ATC system. The occurrence of five "components" in solutions of natural "mauve factor" and kryptopyrrole was instrumental in guiding us to the structure of the factor, but it also illustrates the basic instability of kryptopyrrole, and particularly its spontaneous conversion into various forms. This multiple spot phenomenon is an interesting side issue in our application of ATC to the identification of mauve factor, a side issue which will be reported elsewhere. Conversely, while all five component spots *in situ* can scarcely be identical, their spontaneous development and interconversion, uniquely when pure kryptopyrrole is processed, means this system of related spots can, for brevity, be referred to as kryptopyrrole.

The multiple spot phenomenon could not be attributed to impurities in the kryptopyrrole used (purissimum grade, Aldrich) as its high-resolution mass spectrum matched that reported in the literature[14,15] and indicated a very high purity.

In attempts to clarify further the constitution of kryptopyrrole and of "mauve factor", autotransfer chromatograms were used preparatively, the spot areas excised and eluted with distilled water. On ATC monitoring of the concentrated eluates, however, all of the "mauve factor" components had reverted essentially completely to component 1; exactly the same reversion occurred with the components from kryptopyrrole. Thus far, all methods of eluting these component spots result in interconversions within the general system of five component spots.

While this unusual behavior complicates the chemical study of kryptopyrrole, the exact parallelism exhibited by kryptopyrrole and "mauve factor" throughout these experiments provides additional evidence that they are identical. In fact, it has been impossible to distinguish solutions of "mauve factor" from solutions of kryptopyrrole using a battery of chromogenic, spectrophotometric, electrophoretic, and chromatographic tests (Table I).

HIGH-RESOLUTION MASS SPECTROMETRY

The results at this point seemed to demonstrate adequately that "mauve factor" was, in essence, kryptopyrrole. Having applied optical spectroscopy to the problem (Table I) we turned to mass spectroscopy of highly purified extracts, but found that low-resolution mass spectroscopy was of very little value in this application, due to the interconversions and the presence of isobaric alternatives and stubborn impurities.

In contrast to this, high-resolution mass spectrometry has yielded extremely valuable results when applied to these highly purified extracts and particularly when applied to eluates from preparative autotransfer chromatograms. The direct application of high-resolution mass spectrometry to exhaustively purified extracts did yield the characteristic molecule ion peak (*m/e* 123.106) in samples where "mauve

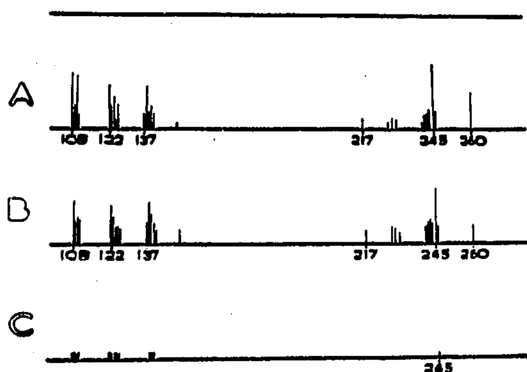

Fig. 2. Mass spectra of eluates from ATC component 1 position. A = synthetic (dikryptopyrryl ether); B = urinary "mauve factor"; C = blank chromatogram.

factor" was present, and equally demonstrated its absence in samples where "mauve factor" was absent. Nevertheless, the procedures used could not be readily generalized to other identification problems in biochemical psychiatry, the procedures were highly specialized and tedious, and much prior knowledge concerning the chemistry of the unknown was essential in order to allow focusing at the appropriate precise mass value.

In the second method of applying high-resolution mass spectrometry to compounds previously assessed by ATC, the ATC is used to actually prepare the specimen for mass spectrometric study. This procedure has been applied to the specific identification of the molecular structure of the components making up the characteristic kryptopyrrole "fingerprint". This work has already revealed that "mauve factor" component 1 and the corresponding spot eluate from kryptopyrrole have the same high-resolution mass spectrum, identical with the main component (*m/e* 260, dikryptopyrryl ether) spontaneously formed from kryptopyrrole in water (Fig. 2). Details including confirmation by deuterium labeling will be presented elsewhere.

From an examination of Fig. 2 it is clear that the mass spectrum of the component 1 spot is sufficiently "clean" and characteristic to enable identification of the compound even if we were unaware of its chemical background. To determine whether the present procedure could be applied without modification to identify other, rather

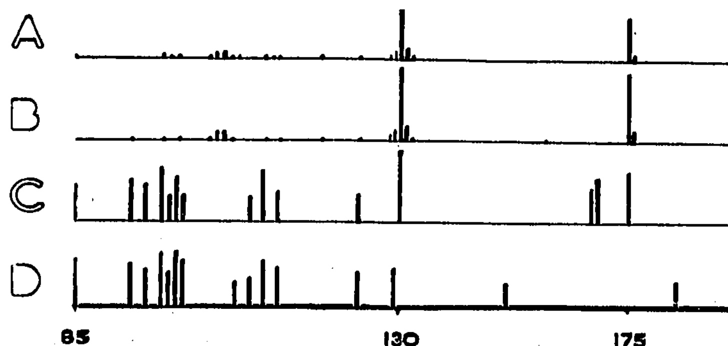

Fig. 3. Mass spectra of eluates from ATC indole-3-acetic acid position. A = synthetic reference compound (indole-3-acetic acid, not chromatographed); B = synthetic indole-3-acetic acid chromatographed and eluted; C = crude urine extract chromatographed and eluted; D = blank chromatogram, eluted.

different structures among urinary metabolites, we selected the indolic acids, indole-3-acetic, and its 5-hydroxy derivative. For each acid, mass spectra of four different specimens were compared, *viz.* of the synthetic compound, the synthetic compound from preparative ATC, the presumptive compound prepared by ATC of crude extracts of human urine and a blank eluate of the appropriate ATC position.

Again, in the mass spectrum of the presumptive urinary indole-3-acetic acid, the peaks are sufficiently sharp and characteristic, and contaminating peaks are so clearly comparable to those in the blank (or are not plausibly derivable from the molecule ion peak) that the direct identification of indole-3-acetic acid would have been feasible without additional information (Fig. 3). In the case of 5-hydroxyindole-3-acetic acid, it would not have been possible to identify its structure from the high-resolution mass spectrum of the presumptive spot eluted from an ATC of crude urine extract, since the urinary metabolites were not adequately resolved at the pertinent ATC position. Perhaps this is to be expected, because this compound, under standard conditions, does not actually enter the paper-partition chromatographic field but remains permanently below the transfer solvent front. Nevertheless, it was possible to establish that the precise molecule ion peak of 5-hydroxyindole-3-acetic acid was in fact present in the presumptive urinary "spot", and was absent from the corresponding ATC blank.

Work under way seeks to extend the application of our unmodified procedure to the identification of the precise molecular structure of each of the other four components of "mauve factor" or kryptopyrrole solutions. More generally, it may be anticipated that combined ATC and high-resolution mass spectroscopy will, in future, have both qualitative and quantitative applications. The unmodified present procedure should be suitable for identifying many neutral and acidic monofunctional pyrroles and indoles in biological extracts. It should also be possible to markedly broaden the series of compounds actually identifiable by this type of ATC, simply by appropriate selection of other TLC and PC subsystems closely matched to the specific problem at hand. We anticipate broadening the applicability of the procedure still further, with extension to GLC → TLC, GLC → PC systems like JANÁK described[3], or to CC → TLC, CC → PC, and finally to three dimensional ATC (GLC or CC → TLC → PC).

Finally, quantification of uniquely identified compounds may, in future, be provided by integrating the ion current[16,17] at a selected precise mass position, after injecting autotransfer chromatogram eluates into the mass spectrometer.

EXPERIMENTAL

Samples

Urinary "mauve factor", presumptive indole-3-acetic acid, and presumptive 5-hydroxyindole-3-acetic acid were extracted from urine using the charcoal adsorption method described in ref. 1, but with methanol replacing the acetone. "Mauve factor" was further purified by CC on charcoal and Fullers' earth, using methanol, or by preparative TLC on silicic acid with diethyl ether, and preparative PC using a 9:1 mixture of benzene and 2% aqueous formic acid. The standards were commercial products: kryptopyrrole (purrissimum grade, Aldrich), indole-3-acetic acid (Regis), and 5-hydroxyindole-3-acetic acid (Nutritional Biochemicals).

Thin-layer chromatography

Standard 5 cm × 20 cm TLC plates were prepared in the laboratory using 47.5 g silicic acid (Mallinckrodt) and 2.5 g plaster of Paris per 100 ml of distilled water and were allowed to come to equilibrium over highly active Davison silica gel after activation, and before use in the autotransfer procedure. The plates were developed with AnalaR diethyl ether, in a tightly sealed chamber.

Transfer step

After the thin-layer plate had been developed and dried, a 6-mm strip at one end of a pre-cut sheet of Whatman No. 1 chromatography paper was allowed to overlap the long side of the thin-layer chromatogram. A matching glass plate was placed over this, and the resulting "sandwich" was secured with spring clips, and inserted into a notched polypropylene framework in an air-tight tank containing sufficient transfer solvent to reach a few millimeters up the side of the "sandwich" opposite the paper strip. The transfer solvent consisted of a mixture of propan-2-ol (Fisher reagent grade) and distilled water in a volume ratio of 66:10. Transfer took place by capillary ascent (*ca.* 90 min, 8 cm). The "sandwich" was then disassembled, and the paper strip allowed to dry in preparation for the paper chromatography step.

Paper chromatography

The transferred strips were placed in an all-glass descending chromatography chamber (Kensco 3030), equilibrated for 40 min and then run for 120 min, using a stabilized "solvent D" system described by REIO. It was essential to follow the details provided in REIO's original paper[18].

Elution

The pertinent spot areas were excised from replicate autotransfer chromatograms and eluted with a minimum volume of distilled water, using the descending chromatography method, with solvent supply from the capillary space between two microscope slides.

High-resolution mass spectrometry

Detailed procedures will be reported elsewhere, but 3 to 8 µl aliquots of the water eluates were examined by direct insertion into a high-resolution double-focussing mass spectrometer, the MS9 manufactured by Associated Electrical Industries.

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