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AUTOTRANSFER CHROMATOGRAPHY IN THE CHARACTERIZATION OF PYRROLES

CHEMISTRY OF MULTIPLE-SPOT PHENOMENA

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SUMMARY

A characteristic pattern of five interconverting "component" zones is obtained on autotransfer chromatography when pure kryptopyrrole (KP) is adsorbed from aqueous solution onto charcoal and then eluted with methanol. Specific oxidation products (lactams) derived from synthetic KP matched four of the five zones. Zone 1 was obtained directly from the α'-hydroxy-α-lactam. Zone 3 was produced directly by the α'-methoxy-α-lactam, while zones 2 and 5 were formed as a consequence of its acid hydrolysis during equilibration and chromatographic development. The formation of the methoxy lactam itself depended upon the presence of added methanol in the extraction process. In the absence of methanol during extraction only zone 1 (sometimes accompanied by zone 4) was formed.

INTRODUCTION

Alkyl monopyrroles commonly yield more than one spot when analyzed by hybrid 2-dimensional (autotransfer) chromatography[1], particularly if small amounts of them have been carried through the usual procedures[2] for extracting such compounds from biological sources. Under standardized conditions, the patterns of multiple spots are characteristic of monopyrroles with certain alkyl substituents, and these patterns can be useful in determining the structures of naturally occurring pyrroles[3]. For example, kryptopyrrole (KP) yields the characteristic pattern of "component" zones numbered 1 to 5 in Fig. 1.

Nevertheless, the components of any one of these chromatographic patterns, while generally attributable to a specific alkyl monopyrrole as the formal parent structure, can scarcely be identical. A further understanding of the composition of the components, and of the mechanism(s) responsible for their appearance, should clarify the rather unusual interconvertibility[4] of such components, and may have implications for analytical procedures used to determine labile naturally-occurring

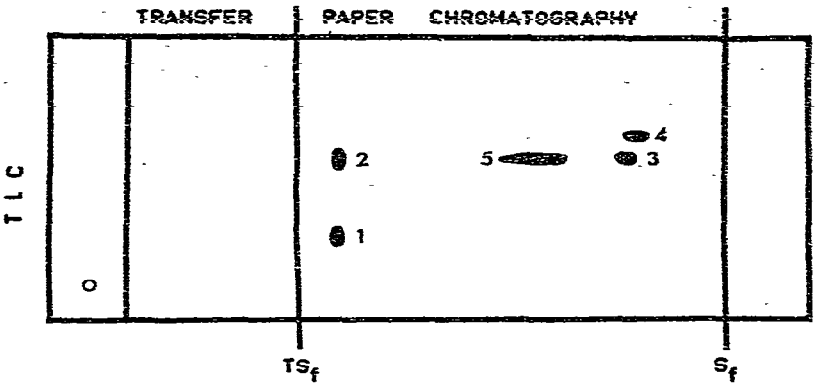

Fig. 1. Characteristic pattern of multiple zones obtained by routine ATC of aqueous–methanolic solutions of KP or hemopyrrole. Material to be studied is applied at the origin (O) and developed on the TLC plate, transferred laterally onto paper, and then further separated by paper chromatography (between the transfer solvent front, TS_f, and the chromatography solvent front, S_f).*

monopyrroles such as KP* or its isomer hemopyrrole[5] and their oxidation products.

There has long been evidence to suggest the presence of oxygen in the natural KP from urine (*i.e.*, elemental analyses[6], infrared spectra[6], synthesis of a simplified model compound[6], structural group analysis by autotransfer chromatography[4], and high-resolution mass spectrometry of the isolated material[1]). An adequate standard or series of reference compounds (*i.e.*, monocyclic oxidation products of KP) and a knowledge of their chemistry were both lacking until Lightner and Crandall's recent characterization[7] of four specific compounds produced from KP by photooxidation. Two of these "synthesised components" from pure synthetic KP, have now been shown by available chromatographic methods, to be isographic with components of naturally-occurring "KP", including some from a case of acute intermittent porphyria[8]. Chromatographic investigations of these model compounds, which are presented in detail in this paper, have helped to clarify the previously puzzling multiple spot phenomena.

EXPERIMENTAL

The structures of four different di-oxy compounds isolated by Lightner and Crandall[7] from the dye-sensitized photooxidation products of synthetic KP are given in Fig. 2; corresponding abbreviations and synonyms are tabulated in the legend of that figure.

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* Recent work[29] indicates that in spite of the excellent resolution of the ATC system, and the isographic behavior of the natural metabolite in co-chromatography with authentic KP oxidation products, these procedures do not rule out the recognized possibility[3,24] that we are dealing at least in part with the corresponding oxidized forms of hemopyrrole (which is a simple side-chain positional isomer of KP). Consequently, while hemopyrrole and its oxidation products behave in a fashion strictly parallel with the corresponding krypto series, it should be understood that, throughout this article, where reference is made to the endogenous metabolic product, "hemo" may be read for "krypto".

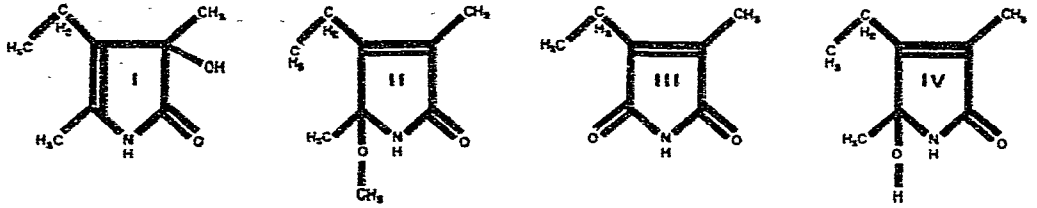

Fig. 2. Structures of four photooxygenation products produced from pure synthetic KP. These structures may be designated as follows, where L stands for "-lactam-2", and the first prefix may be the number or the corresponding Greek letter: I = 3-OH-KPL or β-OH-KPL; 4-ethyl-3-hydroxy-3,5-dimethyl-Δ^4-pyrrolin-2-one; 4-hydroxykryptopyrrolone-5. II = 5-MeO-KPL or α′-MeO-KPL; 4-ethyl-5-methoxy-3,5-dimethyl-Δ^3-pyrrolin-2-one; 2-methoxykryptopyrrolone-5. III = MEM, methylethylmaleimide. IV = 5-OH-KPL or α′-OH-KPL; 4-ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one; 2-hydroxykryptopyrrolone-5.

The synthetic compounds were monitored both directly and after processing through a standard extraction procedure, using the six chromatographic systems listed in Table I.

The routine procedure for system 6 (autotransfer chromatography or ATC[3,9]) involved separation of standard substances and extracts on silicic acid thin-layer chromatographic (TLC) plates followed by the transfer of the separated compounds laterally on to paper using a mixture of isopropanol–water (7:3). The paper strip

TABLE I

CHROMATOGRAPHIC SYSTEMS EMPLOYED

No.	*System*	*Proportions (v/v)*	*Comment and reference*
1	Isopropanol–conc. ammonium hydroxide–water; Whatman 3MM paper	20:1:2	ref. 25
2	8% sodium chloride in 1% acetic acid; Whatman 3MM paper		ref. 26
3	Conc. ammonium hydroxide–water–diisopropyl ether; Whatman 3MM paper	1:100:10	Aqueous phase used (ref. 2)
4	Benzene–2% formic acid; Whatman No. 1 paper	10:1	Tank geometry and equilibration methods were exactly as specified for solvent D (ref. 27)
5	Diethyl ether; silicic acid plates		A slurry of silicic acid (Malinckrodt, 47.5 g) and plaster of Paris (2.5 g) in water (100 ml) was spread on glass plates (Desaga TL spreader, 0.5 mm setting), then dried at room temperature, activated at 105° for 40 min., and stored in a desiccator
6	A two-dimensional combination of systems 4 and 5, referred to as autotransfer chromatography (ATC)[3,9].		

was then equilibrated with and chromatographically developed in the descending fashion in solvent system 4. Any responses of the zones to short- or long-wavelength UV light were noted before chromogenic development with Ehrlich's spray reagent (1 g *p*-dimethylaminobenzaldehyde in a mixture of 5 ml conc. HCl and 45 ml water). Co-chromatography was carried out in those cases where the photooxidation products appeared to match components of KP treated in the same way.

Studies of the various standards and extracts occurred in four phases. In the first phase, the behavior of four model compounds obtained by photooxidation was studied, without prior treatment, using all six of the chromatography systems, without modification. In the second phase, the ATC system was modified systematically and complementary procedures added where needed to clarify a point. In the third phase, the ATC system was kept constant, but the model compounds were not applied directly to the thin layer, but were separately dissolved in water and carried through the routine mild procedure[2] used in different laboratories[10,11] to isolate "natural KP". In the fourth phase, the results from the preceeding phases were used to develop some preliminary extraction procedures which would avoid the formation of secondary products, and thus allow the determination of the essential form(s) of "natural KP" in various body fluids. Details of these phases of the work are found in sequence in the next section, coordinated with related results and discussion.

RESULTS AND DISCUSSION

Throughout the work reported here, the R_F values for the various component zones of kryptopyrrole were as published earlier[3]. The characteristic multiple-spotting phenomenon was not restricted to this particular pyrrole, but was seen (in a slightly modified and definitely offset form) when aqueous methanolic solutions of 2,4-dimethylpyrrole or of 2,4-dimethyl-3-acetylpyrrole were chromatographed in the same systems. Most recently, hemopyrrole (2,3-dimethyl-4-ethylpyrrole) has been found to yield an ATC pattern of five zones indistinguishable from that produced from its isomer KP[5].

Phase I

Of the four synthetic oxidation products of KP, one (compound III, methylethylmaleimide) failed to react with Ehrlich's reagent either directly or after processing through the routine extraction procedure. Consequently, it would appear to have little to do with the characteristic ATC pattern and it has not been considered further.

The remaining three synthetic compounds all reacted with Ehrlich's reagent to yield colours, whose hue, intensity and rate of development were essentially identical, both amongst themselves and with respect to extracts from urine or aqueous solutions of pure synthetic KP passed through the routine extraction procedure. Since there is no free α position in these compounds, this striking colour development is contrary to existing theory[12] and appears to be a clear-cut class exception.

When these three synthetic compounds were chromatographed in systems 1, 2 and 3, they exhibited the same R_F values and the same negative response to UV (254 and 366 nm) as the authentic zones from the KP extracts. However, using systems 4, 5 or 6, compound I yielded a single zone, different from any of the usual five. The

discrimination of this new spot from those in the characteristic pattern was even more obvious when formic acid was omitted from system 6.

When compounds II and IV were applied directly to the TLC plate they corresponded perfectly with four of the zones shown in Fig. 1. That is, without prior treatment (*i.e.*, adsorbtion, extraction, etc.), compound IV produced a single ATC zone 1 and compound II produced three ATC zones 2, 3 and 5. Clearly, neither of these compounds produced zone 4. Direct co-chromatography of compounds IV or II with extracts from aqueous solutions of natural or synthetic KP, showed isography with the appropriate zones.

Phase II

It was nevertheless curious that three zones should be obtained on direct ATC of pure compound II. Since these were all at the same R_F in the TLC dimension and one (zone 5) was elongated between the other two in the paper dimension, it seemed likely that conversion from one form to another was occurring during chromatography. This was investigated in six steps:

(i) The TLC and transfer steps were eliminated by spotting directly onto the paper. This had no effect on the results; consequently the appearance of zones 2, 3 and 5 is inherent to the last (*i.e.*, paper chromatographic) step of the ATC. This step was next studied in detail.

(ii) The formic acid used in the paper chromatographic step was replaced with water for both the equilibration and development. Compound II then produced only zone 3. Other acids were substituted for formic: when acetic acid was used, compound II again produced all three zones. Consequently, the formation of zones 2 and 5 is due to the action of added acid, but not specifically formic acid. The results with acids of lower pK_a values were complicated, and will be reported later.

(iii) Acid was eliminated from the equilibration but not from the chromatographic development (to do this, the regular, acid solvent 4 had to be artifically advanced to just wet the spots on the starting line immediately after equilibration in a formic acid-free atmosphere). Only zones 3 and 5 appeared, showing that equilibration in the acid atmosphere was responsible for the appearance of zone 2.

(iv) Compound II was applied directly to paper and developed with solvent 4 in two dimensions (diagonal chromatography). In this method, deviations of zones from the diagonal indicate chemical conversions taking place during the actual development[13–15]; in the case of compound II, zones 2 and 3 were on the diagonal, but zone 5 was represented by a streak in the form of a right-angle linking zones 2 and 3. Clearly, zone 5 is being formed gradually from compound II during liquid-phase development with solvent 4.

(v) Compound II was pretreated in a test tube with formic acid solution at the same concentration and temperature as used in the ATC system and then chromatographed using the entire ATC procedure (but without formic acid). Only zone 1 appeared. Since compound IV yields zone 1 directly, and compound II yields zone 3 directly, these results indicate that compound IV is formed from compound II under these mild conditions.

(vi) Zones 2 and 5 were eluted (using water) from a regular ATC. When these eluates were re-chromatographed they both yielded only zone 1. Consequently, these zones are chemically identical with zone 1. Zones 2 and 5 therefore are formed

from compound II through vapour-phase and liquid-phase acid-catalysed "hydrolysis" of the methoxyl group attached to the α' carbon atom, C_5.

Phase III

Even though one of the pure synthetic compounds yielded three zones, there was considerable specificity in the relationships between the ATC zones and particular synthetic oxidation products of this pyrrole, when these were spotted without prior treatments. In particular, none of the synthetic compounds yielded zone 4, and compound I gave none of the five zones. In sharp contrast to this, there were remarkable interconversions or "scrambling" when the synthetic compounds were individually dissolved in water and carried through the routine analytical procedure[2] updated to substitute methanol for acetone throughout. Following this routine charcoal–methanol processing, all five typical ATC zones of the KP series were seen, whether compound IV, compound II, or even compound I was processed.

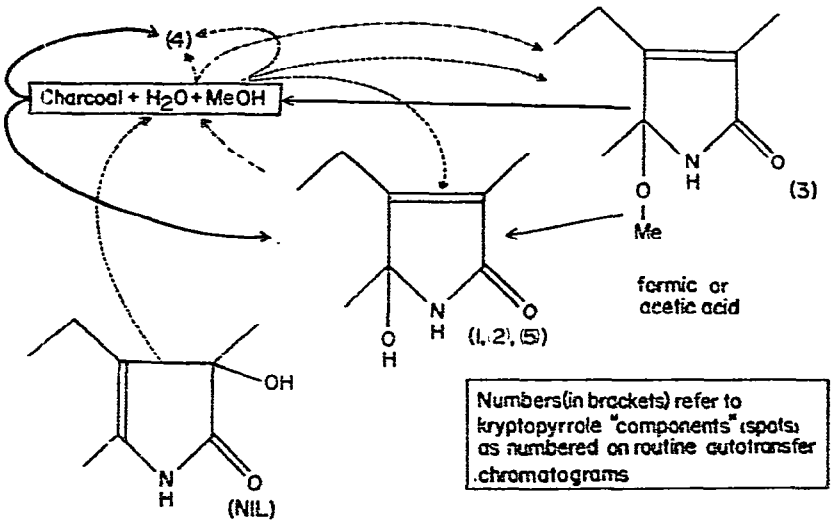

Fig. 3. Relationships between chemical structures, chemical interconversions during extraction and chromatography, and the chromatographic "components" (multiple zones) from KP solutions.

These results further illustrate the ready interconvertibility of different components of the KP series. They also suggest that zone 4, like all the others, is due to some substance derived from compound IV. Interestingly, while compound I is not produced from any of the other synthetic compounds during extraction, it is itself converted by this process to yield all five of the zones. The known structures and conversions involved in the "scrambling" phenomenon are summarized in Fig. 3.

From these results it is clear that only two compounds (II and IV) are responsible for four out of the five zones (or so-called "component spots") on ATCs' of urinary KP or aqueous solutions of synthetic KP, and these two compounds are easily interconverted using water or methanol, especially in the presence of a catalyst. Such processing with methanol converts compound IV to the corresponding methoxy

compound II. Since compound II depends on the presence of methanol in the extraction or processing system, it too is essentially a product of the analysis and the main component was therefore concluded to be compound IV.

Phase IV

As predicted from our conclusion that zones 2, 3 and 5 were due to reaction of the native compound IV (and possibly the unknown chemical form responsible for zone 4) with methanol used in the extraction, our pilot study on extraction of blood with ethanol (or ethanol–ether) showed that zones 2, 3 and 5 were indeed uniformly absent. On the other hand zone 1, sometimes accompanied by zone 4, was clearly demonstrated in the blood of those individuals excreting "natural KP" in their urine. Neither zone 1 nor 4 was demonstrated in blood from persons negative for the urinary metabolite. When cerebrospinal fluid from a patient who excreted the metabolite was extracted with ethanol and analysed by ATC, zone 1 was again detected.

These results constitute the first demonstration of "natural KP" in body fluids other than urine (see Table II for details); they identify this metabolite not merely as some "form" of KP, or something "attributable" to KP, but as the specific α'-hydroxy, α-lactam compound IV (or probably the β,β' sidechain positional "hemo" isomer[5] of compound IV). The clinical chemical results provided further confirmation that zone 1 represents the "basic" or "parent" compound of the pyrrole-lactam series.

TABLE II

SOME PRELIMINARY ANALYTICAL METHODS FOR NATURALLY OCCURRING KRYPTOPYRROLE COMPONENTS, USING ETHANOL

Tissues, blood or other body fluids were extracted, the combined extracts lyophilized to 0.5 ml, then separated by ATC. The design of these exploratory extraction procedures also took into account the difficulties previously experienced in attempting methanol extraction of KP from tissues, and the recent success[28] using ethanol to extract pyrroles from tissues.

Source	*Method*
Tissues	1 g was homogenized and extracted twice with 2–3 ml ethanol
Blood	8 ml were extracted twice with 20 ml ethanol each time, and once with 10 ml ethanol
Other body fluids	9 ml were mixed with 20 ml ethanol and extracted twice with 20 ml diethyl ether

Additional evidence that compound IV or its hemo isomer is the key substance of the series was obtained by ATC monitoring of KP extracts prepared in different ways from clinical urine specimens; the spot common to virtually all positive extracts was found to be zone 1. The only other component seen frequently and even where methanol was complete avoided, was zone 4.

GENERAL DISCUSSION

The study in biological fluids of certain pyrrolic substances is rendered very difficult because of the multiple-spotting phenomenon during chromatography and

the interconvertibility of some substances. What is clear however, is that the so-called components of the KP pattern are really oxidized forms of KP.

The present work has demonstrated that four out of the five "component" zones found when solutions of KP are chromatographed are due to two oxidation products: the α′-hydroxy and α′-methoxy α-lactams of KP. The hydroxy lactam (IV) provided zone 1 in a straightforward manner, but the methoxy lactam (II) yielded zone 3 directly, and zones 2 and 5 by acid "hydrolysis" during equilibration and development. The nature of the remaining zone 4 could not be clarified in this study although there is some evidence to suggest that it, like all the other "components" of polar solutions or extracts of KP, is an oxidized form of the KP molecule. A subsequent paper will deal with the identification of the structure and mechanism of formation of zone 4, along with some considerations of the organic chemical reaction mechanisms and molecular biology relating to the entire set of five interconverting component zones.

These multiple-spotting phenomena were found to be further complicated by the effects of prior extraction. In the case of the synthetic KP, which is also the case for the natural metabolite, methoxy lactam formation appears to depend on the presence of methanol in the analytical system. As a result zones 3, 2 and 5 are all secondary products. The facile incorporation of methoxy groups from solvent methanol has been observed independently by Lightner *et al.*, during the photochemical oxygenation of synthetic KP[16]. In an aqueous medium the expected member of the lactam family from KP would be the α′-hydroxy lactam, and in a methanolic solution this would be converted to the corresponding α′-methoxy lactam. Addition of water to the methanol solution would tend to convert the methoxy compound back to the hydroxy form. This ready interconversion has a definite parallel in the reactions already reported[17] for some naturally-occurring homologues of the KP lactams, the propentdyopents.

The relationship between compound IV (α′-hydroxy-kryptopyrrole-α-lactam), zone 1, and "dikryptopyrryl ether"

In previous work[1] it was found that the identical mass spectrum was obtained on eluting zone 1 from ATC's of "natural KP" or from ATC's of pure synthetic KP in aqueous solution. This spectrum was that expected for "dikryptopyrryl ether", a compound first described by Fischer *et al.*[18,19]. The mass-spectral results were quite clear-cut, but as noted previously[4], it was difficult to see why a simple ether should be found in an area of the chromatogram characteristic of far more polar compounds, especially amides, lactams and diols. In a recent re-investigation[20] of the structure of the so-called "dikryptopyrryl ether" it was established that it is not an ether, but rather an isobaric alternative structure, in fact the lactam, α′-kryptopyrryl-kryptopyrrole-α-lactam. This accounts for its quite polar character noted as an apparent paradox in our earlier chromatographic studies.

While the revision of the chemistry of this "ether" makes it clear that we are dealing in all cases with lactams of basically the same structure, the question remained as to why a dimeric lactam was found mass-spectrometrically, while all the other evidence points to the monomeric α′-hydroxy lactam as being responsible for zone 1. The highly reactive nature of the α′-hydroxy KP lactam (compound IV) and especially its tendency to dimerize, rather than any pre-existence of the dimeric "dikryptopyrryl

ether" in zone 1 of the chromatogram, may well have been responsible for our earlier identification[1] of the mass spectrum of "dikryptopyrryl ether" in eluates of this zone. Dimerization of such pyrrolenones has been reported[21,22], and the products could yield the mono-oxygenated dipyrrolic lactam (formerly called the ether) by a reaction within the mass spectrometer, or possibly by a photoreduction[23].

The key (or basic) component of "natural kryptopyrrole"

All of the experimental evidence assembled to date strongly suggests that the key substance in naturally occurring KP is the α′-hydroxy-α-lactam, on the grounds that it is: (a) the most abundant component; (b) the component most frequently observed; (c) the component least dependent on the analytical methods used (zone 3 depends on added methanol, and 2 and 5 depend on added organic acids); (d) the dominant one of two components found in blood; (e) readily formed from the other lactams by the action of water at room temperature, hence the most likely form to be expected in body fluids and tissues.

It is concluded therefore that the key structure among the components of natural KP is that illustrated in Fig. 2 as number IV (or its hemo isomer). The formal chemical name of this KP hydroxy lactam structure is 4-ethyl-5-hydroxy-3,5-dimethyl-Δ^3-pyrrolin-2-one. The corresponding hemo isomer which must also be considered[5] is 3-ethyl-5-hydroxy-4,5-dimethyl-Δ^3-pyrrolin-2-one.

This laboratory has pointed out[8,24] the difficulty of excluding the possibility that some or all of the so-called natural KP might be the corresponding hemo isomer. While our recent work shows that hemo and krypto series of lactams are isographic in the existing chromatographic systems including ATC, a new system of higher resolution has just been developed[5] to answer the question of what isomer(s) may be present naturally; the procedure will be reported elsewhere in detail.

CONCLUSIONS

The present basic and applied work on the formation and interconversion of multiple zones in the ATC of KP allows the following conclusions.

(i) None of the five zones represent unchanged KP, but all are oxidized forms (lactams).

(ii) Two of the zones are derived from a methoxy lactam by acid-catalysed reactions during equilibration and chromatographic development, respectively.

(iii) This methoxy form is derived from the α′-hydroxy-α-lactam (or native metabolite) by reaction with methanol during extraction.

(iv) In the absence of added acids and methanol only two of the five zones appear: the α′-hydroxy-α-lactam corresponding to KP, and the minor, as yet unidentified zone 4.

(v) Analytical procedures designed to prevent the formation and interconversion of secondary products demonstrated the presence of the α′-hydroxy-α-lactam in the blood or cerebrospinal fluid of those persons excreting natural KP (lactam adducts) in their urine.

It is concluded that "natural KP" as well as synthetic KP in dilute aqueous solutions, exists primarily as the α′-hydroxy-α-lactam; and that adduct exchange reactions account for most of the formerly puzzling interconversion, multiple-spotting and apparent "heterogeneity" of this substance.

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