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# SIMULTANEOUS LABELLING WITH STABLE AND RADIOACTIVE ISOTOPES IN DRUG METABOLISM STUDIES

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**Abstract**—Stable isotope labelling facilitates structure elucidation and identification of drug metabolites. Simultaneous labelling with $^{13}C$ and $^{14}C$ at the same position of a molecule enables the critical examination of metabolite patterns. Even minor amounts of metabolites can immediately be isolated, structurally assigned and quantitatively estimated. Thus, double labelling with stable and radioactive isotopes is at present the most convenient method for studying parameters of drug metabolism.

THE APPLICATION of mass spectrometry to structure elucidation of drug metabolites is often seriously complicated by impurities. It is difficult to decide whether the fragments in the mass spectrum belong to the metabolite or possible impurities. This necessitates further purification, as otherwise the mass spectrometric analyses cannot be successfully applied. These difficulties can be overcome by specific labelling which marks the ions of a drug as well as its metabolites in the fragmentation patterns. This can be achieved by artificially formed isotope clusters of the parent drug using stable isotope labelling. The great advantages of this approach have recently been demonstrated,[1-2] but, in spite of this quantitative data from mass spectrometric analyses for the pharmacokinetics of structurally undefined compounds are not generally available.

During the development of a new therapeutic substance much quantitative data must be obtained in order to determine the range of its biological activity. Such data are best provided by using radioactive labelled compounds. Thus radioactive drugs and their metabolites can be monitored in biological samples at trace levels. At present, no analytical method can replace the accuracy and low detection limits of radioactive measurements.

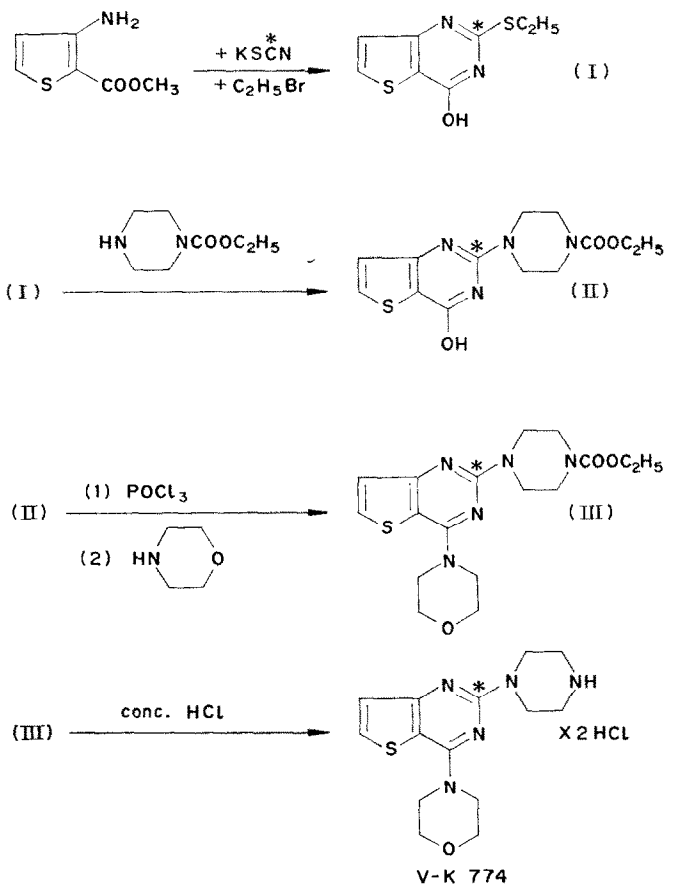
These considerations led to the development of double labelling with stable and radioactive isotopes. This offers simultaneous estimation of pharmacokinetics (radioactive labelling) and structure elucidation (mass spectrometry). Thus, metabolite patterns routinely achieved in the course of distribution and excretion studies can be immediately subjected to mass spectrometry. $^{14}C$ content of even a highly radioactive compound cannot be used for detecting the resulting ions.

Several combinations of stable and radioactive isotopes can be considered. However, the use of $^{13}C$ implies the advantage of simultaneous labelling with $^{14}C$ in the "same" position of a drug. Therefore the synthesis can be easily accomplished. The double labelled drug can be obtained in a "single" synthesis by mixing the appropriate starting materials labelled with $^{13}C$ and $^{14}C$. 4-Morpholino-2-piperazino-thieno [3·2-*d*] pyrimidine (V-K 774) was taken as an example to demonstrate this approach.

The fate of this inhibitor of thrombocyte aggregation was studied in the rat.[1] This paper deals with some qualitative and quantitative biological aspects of the metabolism of V-K 774.

## MATERIALS AND METHODS

*Synthesis of $^{13}C$ and $^{14}C$ labelled V-K 774.* $^{13}$C-KCN (Merck, Sharp & Dohme, Montreal) and $^{14}$C-KCN (New England Nuclear Corp., Boston, Mass.) were mixed prior to the chemical synthesis and converted to the corresponding KSCN. The synthesis was performed with this mixture (scheme 1).[3] A yield of 1·775 g V-K 774 with 60% enriched $^{13}$C and with $^{14}$C of a specific activity of 0·52 $\mu$Ci/mg were obtained as V-K 774 × 2 HCl × 3 $H_2O$.




SCHEME 1.

*Administration of $^{13}C$–$^{14}C$ V-K 774.* 100 mg/kg of 2-$^{13}$ C-$^{14}$ C-V-K 774 × 2 HCl × 3 $H_2O$ were administered orally in 4 ml aqueous solution to female fasted adult rats (strain FW 49 Biberach). The animals were housed in metabolism cages during the experiment. Urine and faeces were collected separately for 24 hr periods up to 120 hr

after drug administration. The rats received water *ad lib.* and standard food (Altromin R, Altrogge, Lippe), ground and suspended in water.

*Measurement of radioactivity.* 0·1 ml of a urine sample and/or a urine extract was mixed with 15 ml of scintillation fluid (Diotol*) and counted in a liquid scintillation counter (Packard Model 3380).

Freeze-dried faeces were pulverized and 1 ml of 30% $H_2O_2$ was added to 50 and 100 mg portions of this material. The mixture was decolorized overnight at 40°. Two ml of Digestin (E. Merck, Darmstadt) were then added and the sample maintained at 40° overnight. Subsequently each sample was made up to a volume of 25 ml with Diotol. Five ml of this mixture were mixed with 10 ml of Diotol and counted in a liquid scintillation counter.

*Isolation of urine metabolites.* Approximately 100 ml of urine were mixed for 0·5 hr with about 5 g Amberlite XAD-2 resin (Serva, Heidelberg) using a magnetic stirrer and then washed with water. The resin was then eluted with about 100 ml of methanol and the eluate concentrated under reduced pressure. The concentrated solution was chromatographed on thin-layer plates.

*Thin-layer chromatography.* Thin-layer chromatography (TLC) was performed on precoated Silica gel plates ($F_{254}$, 0·25 mm layer-thickness, E. Merck, Darmstadt). The solvent systems: benzene–ethyl acetate–methanol–concentrated ammonia (50:35:15:5, v/v) and: chloroform–ethanol–acetic acid (85:10:5, v/v) were used for two-dimensional chromatography. The compounds were located by their u.v.-absorption and visualized by Dragendorff spray reagent (modification according to Munier[4]).

*Mass spectrometry.* A CH-5 mass spectrometer (Varian-MAT, Bremen) equipped with a direct inlet system was used. All samples were introduced directly into the ion source and the mass spectra were recorded using 70 and 12 eV, respectively. The Silica gel scraped from the TLC-plates contained 10–50 $\mu$g of each metabolite. The metabolites were eluted with methanol. The concentrated eluate was then subjected to mass spectrometry.

*Quantitative determination of metabolites.* For the quantitative determination of V-K 774 and its metabolites, the Silica gel was scraped from the two-dimensionally developed TLC-plates, corresponding to the area of the metabolites located by autoradiography. The Silica gel was transferred to counting vials, mixed with 15 ml scintillation cocktail (Diotol) and counted.

## RESULTS

*Structure elucidation by mass spectrometry.* Comparison of the mass spectra of the labelled and unlabelled V-K 774 in Fig. 1 clearly demonstrates the "marking" effect of $^{13}C$. The breakdown of this compound is dominated by known fragmentations of the piperazine moiety leading to the formation of the fragments M–30 (I), M–42 (II) and M–56 (III).[1, 5]

As outlined elsewhere[1] the loss of 68 a.m.u. (IV) from the parent ion by rearrangement of three hydrogen atoms is highly specific for the unchanged piperazine substituent.

The mass spectrum in Fig. 2 can be immediately assigned to metabolite M 1 of V-K 774, according to the characteristic ion doublets. This metabolite was isolated

* Composed of 780 g naphthalene, 50·25 g PPO, 0·975 g POPOP, 3·75 l. toluene, 3·75 l. dioxane and 2·25 l. methanol.

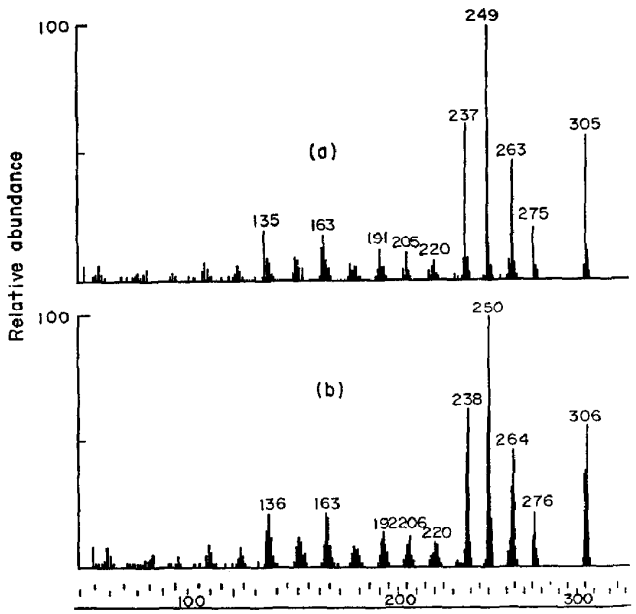
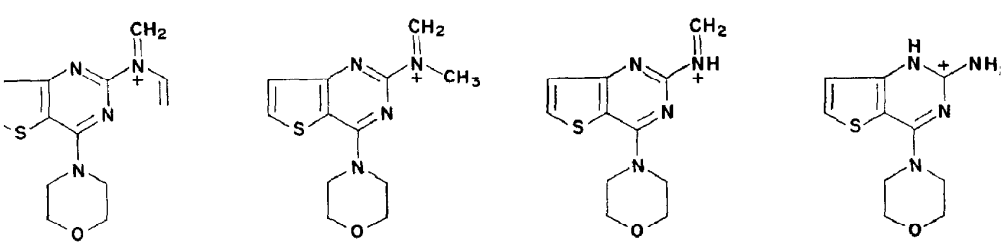



FIG. 1.(a) Mass spectrum (70 eV) of unlabelled V-K 774 (4-morpholino-2-piperazino-thieno [3·2-d] pyrimidine). (b) Mass spectrum (70 eV) of $^{13}C$-labelled V-K 774 (4-morpholino-2-piperazino-thieno [3·2-*d*] pyrimidine 2-$^{13}C$).

by subjecting the untreated rat urine to two-dimensional thin-layer chromatography. The corresponding spot was scraped off, eluted with methanol and analysed by mass spectrometry without any further purification. The increase in mass compared with the parent compound suggests an additional acetyl group in the metabolite (42 a.m.u.). This is affirmed by the mass differences of 72 (30 + 42) a.m.u., 84 (42 + 42) a.m.u.




and 98 (56 + 42) a.m.u. between the molecular ion *m/e* 347/8 and the fragment ions *m/e* 275/6, *m/e* 263/4 and *m/e* 249/50, respectively, indicating a *N*-acetyl piperazin substituent. In addition, the loss of 85 and 86 a.m.u. from the parent ion is observable but no ion corresponding to the elimination of 68 a.m.u. As rationalized in scheme 2, this fragmentation can be easily explained with the structure of a *N*-acetylated V-K 774. The less abundant ion doublet *m/e* 333/4 suggests a mixture with the corresponding *N*-formyl compound (M 2).

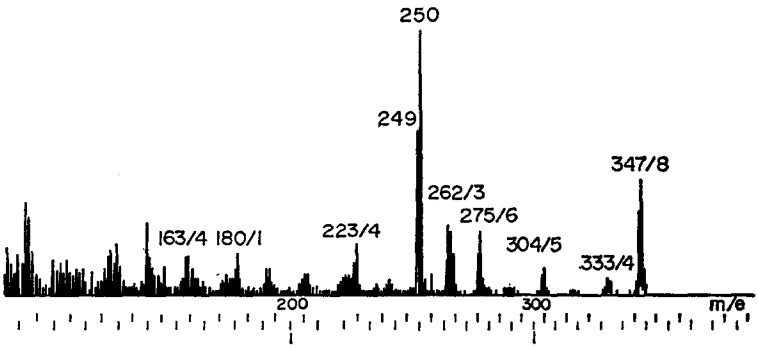
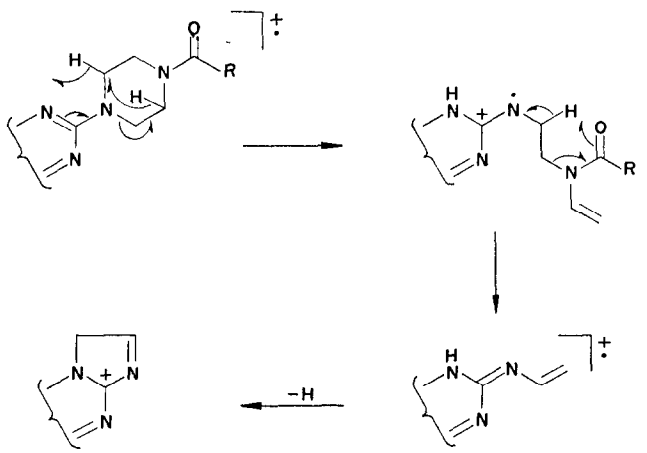



FIG. 2. Mass spectrum of metabolite M 1 (70 eV).

The more complex mass spectrum in Fig. 3 represents metabolite M 7. Compared with the parent compound the molecular ion *m/e* 365/6 shows an increase of 60 a.m.u. that can be interpreted as one additional acetyl group (42 a.m.u.) plus one molecule of water (18 a.m.u.). Several fragment ions, i.e., *m/e* 293/4 (M - 72), *m/e* 281/2 (M - 84), *m/e* 280/1 (M - 85) and *m/e* 267/8 (M - 98), indicate clearly that the metabolite possess




SCHEME 2.

a *N*-acetylated piperazine substituent. Thus, the remaining elements of water must be located in a different part of the molecule. This is supported by the assumption that the loss of 31 a.m.u. from the parent ion forming the fragment ion *m/e* 334/5 involves the removal of a $CH_2OH$-group. Such elimination is not observed with metabolites carrying an unchanged morpholine substituent in addition to a piperazine ring in position two of the thienopyrimidine. Therefore biotransformation of the morpholine ring itself is suggested. The breakdown is best explained in terms of a ring opening to the corresponding diethanol amino substituent. This is for example suggested by the fragmentation processes summarized in scheme 3.

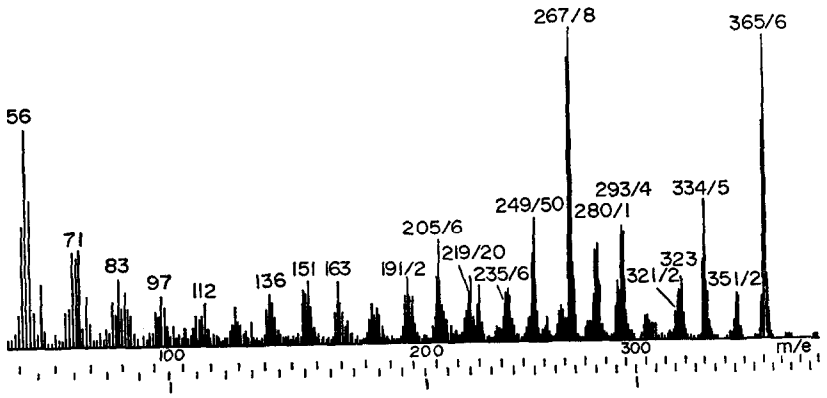
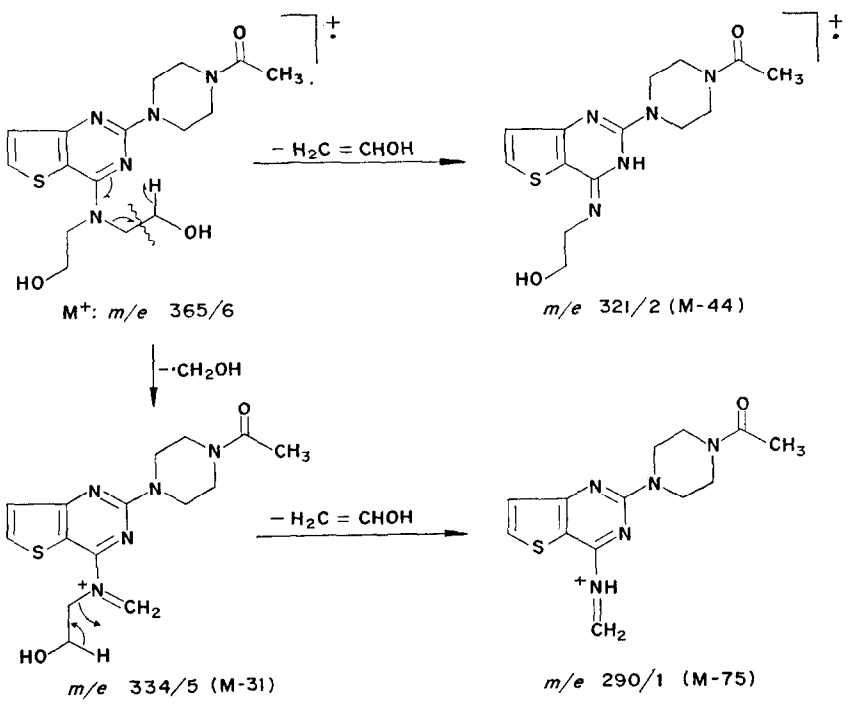
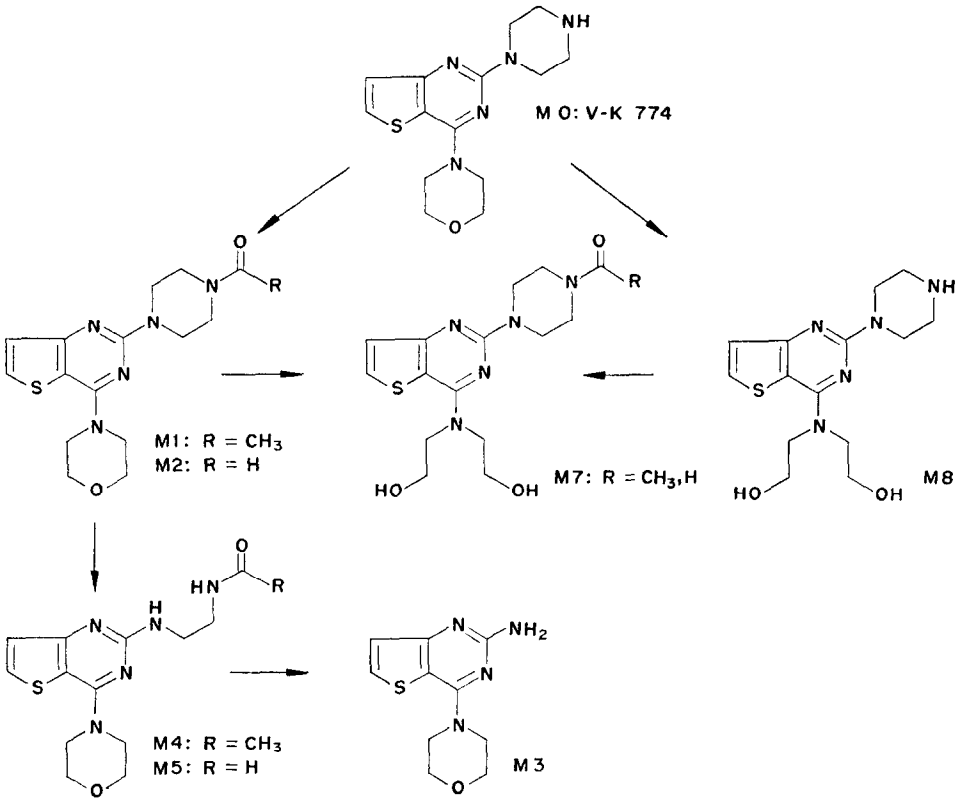



FIG. 3. Mass spectrum of metabolite M 7 (70 eV).

Removal of a $CH_2OH$-group by an $\alpha$-cleavage forms an immonium ion which triggers the elimination of the second hydroxy ethyl-side chain under hydrogen rearrangement. This gives rise to the ion *m/e* 290/1. Elimination of one of the hydroxy ethyl substituents from the molecular ion by a McLafferty rearrangement is indicated by the ion *m/e* 321/2. As can be recognized from ion *m/e* 351/2 in Fig. 3 the sample containing M 7 should consist of a mixture with the corresponding *N*-formyl metabolite.




SCHEME 3.




SCHEME 4.

The structures of several other metabolites of V-K 774 were elucidated in this way by mass spectrometry according to their characteristic isotope distribution. The chemical structures of these metabolites are presented in scheme 4 according their

TABLE 1. EXCRETION OF RADIOACTIVITY

| Hr | Exp. I | Exp. II |
|---|---|---|
| Urine | | |
| 0–24 | 47·1 ± 5·4 | 46·3 ± 3·8 |
| 24–48 | 4·9 ± 1·3 | 3·3 ± 0·8 |
| 48–72 | 1·1 ± 0·6 | 0·8 ± 0·3 |
| 72–96 | 0·3 ± 0·1 | 0·5 ± 0·2 |
| 96–120 | 0·2 | 0·4 |
| Urine | 53·6 ± 5·9 | 51·3 ± 4·2 |
| Faeces | | |
| 0–24 | | 25·4 ± 5·3 |
| 24–48 | 43·5 ± 5·1 | 21·5 ± 6·7 |
| 48–72 | 6·8 ± 2·5 | 6·2 ± 2·4 |
| 72–96 | 1·4 ± 0·5 | 1·4 ± 0·3 |
| 96–120 | 0·4 | 0·8 ± 0·3 |
| Faeces | 52·1 ± 2·7 | 55·3 ± 5·2 |
| Urine + faeces | 105·7 ± 4·1 | 106·6 ± 6·6 |

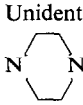
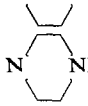
postulated biotransformation pathways. It should be mentioned, however, that some metabolites remain unidentified.

*Excretion of radioactivity.* Recovery of radioactivity after oral administration of $^{13}C$–$^{14}C$ labelled V-K 774 is shown in Table 1.

Recovery of radioactivity and standard deviation (S.D.) in per cent of radioactivity administered after oral administration of 100 mg/kg $^{13}C$–$^{14}C$ V-K 774 × 2 HCl × 3 $H_2O$ to rats. In experiment I, ten and in II, twenty animals (two rats per metabolism cage) were employed. No S.D. was calculated for very low radioactivity excretion. In experiment I no faeces were eliminated for 0–24 hrs after administration.

*Quantification of metabolite patterns.* 100 mg/kg of $^{13}C$–$^{14}C$ labelled V-K 774 × 2 HCl × 3 $H_2O$ were given orally to twenty rats. The 24 hr urine contained 46·3 per

TABLE 2. QUANTITATIVE DISTRIBUTION OF THE METABOLITES IN THE ORIGINAL RAT URINE

| Spot | Metabolite | Chemical structure $R^1$ | Chemical structure $R^2$ | Radioactivity in urine (%) I | Radioactivity in urine (%) II |
|---|---|---|---|---|---|
| 5 | M 1 |  |  | | |
| 6 | M 2 |  |  | 1·9 | 1·8 |
| 7 | M 3 | $NH_2$ |  | | |
| 11 | M 0 |  |  | 29·2 | 30·0 |
| 14 | M 4 | $NH–CH_2–CH_2–NH–CO–CH_3$ |  | 0·5 | 0·6 |
| 15 | M 5 | $NH–CH_2–CH_2–NH–CHO$ |  | 0·4 | 0·4 |
| 18 | M 6 | Unidentified | Unidentified | 0·6 | 0·9 |
| 22 | M 7 |  $R = CH_3, H$ | $N(CH_2–CH_2OH)_2$ | 1·5 | 1·7 |
| 23 | M 8 |  | $N(CH_2–CH_2OH)_2$ | 1·2 | 1·8 |
| 25 (origin) | | | | 34·2 | 31·0 |
| All other spots | | | | 21·7 | 24·2 |
| Areas A–P | | | | 8·4 | 7·1 |
| Σ | | | | 99·6 | 99·5 |

100 per cent of radioactivity quoted is equivalent to 46·3 per cent of the administered dose (cf. Table 1). Spots are designated according to Fig. 4.

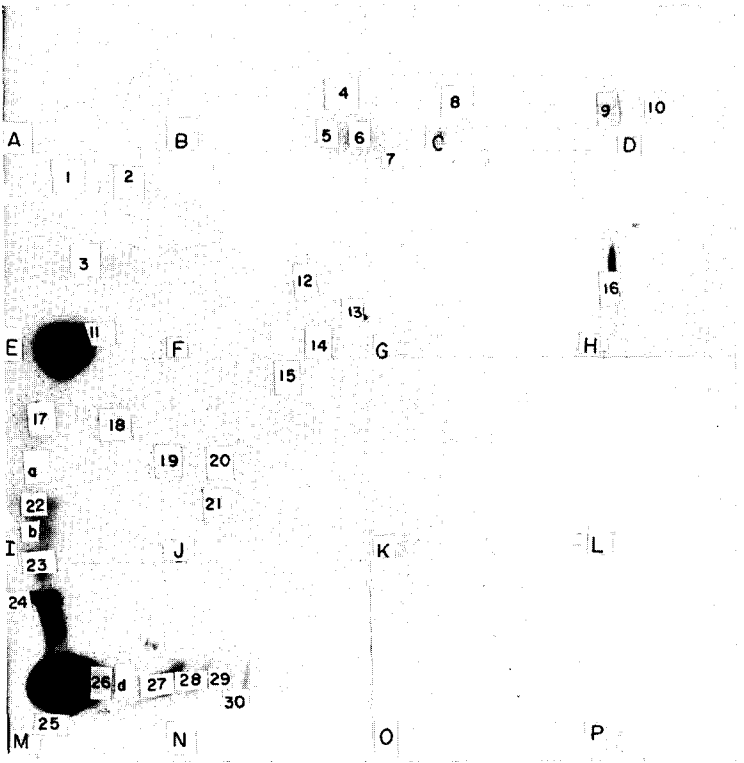



FIG. 4. Autoradiogram of a two-dimensional thin-layer chromatogram of untreated urine. Solvent systems employed: first dimension (ascending) benzene–ethyl acetate–methanol–conc. ammonia (50:35:15:5, v/v) and second-dimension (from the left to the right) chloroform–ethanol–acetic acid (85:10:5, v/v). For the determination of the quantitative distribution of the metabolites (Table 2) the spots were numbered 1–30. Areas a–d are designated with regard to the tailing between the origin and areas 17 and 30, respectively. Capital letters A–P refer to squares on the autoradiogram necessary for total determination of radioactivity on the TLC-plates.

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cent of the 260 $\mu$Ci administered (Table 1). Two samples from this fraction, each 200 $\mu$l (30 nCi) were chromatographed two-dimensionally on TLC-plates (Fig. 4) and the distribution of the metabolites was determined (Table 2).

As indicated in Table 2 unchanged V-K 774 (M O) was the most abundant product in urine. As could be expected considerable quantities of radioactivity remained at the origin of the two-dimensional thin-layer chromatogram. All known metabolites had a much lower concentration. M 1, M 2, M 3, M 4 and M 5 were present in minute quantities. M 1, M 2 and M 3 were poorly separated in untreated urine. However, this approach was suitable for the quantitative determination of these minor urine metabolites. TLC of the untreated urine guaranteed only minimal losses of material. Very small amounts of metabolites might be retained at the origin of the TLC-plates.* One might expect, however, that conjugates of the known metabolites represent a part of this material, for instance of metabolite M 7 and M 8.

The urine had to be further purified by a simple Amberlite XAD-2 elution before structure elucidation of metabolites by mass spectrometry. However, even without this purification step, the structure of M 1 and M 2 were indicated in the mass spectrum of the eluate mixture corresponding to areas 5 and 6 in Fig. 4. This assignment could be made from the typical ion distribution of the $^{12}C$, $^{13}C$ doublets in the mass spectra (Fig. 2).

The faeces contained about 50 per cent of the radioactivity administered. Therefore the material was extracted with methanol in a soxhlet extractor. Only one-half of this radioactivity could be extracted and consisted of very small amounts of the metabolites M 1, M 2, M 3, M 4 and M 5, whereas M 0 represented one tenth of this radioactivity. Two-tenths of the activity were detected as M 8, which was present in much higher concentration than in urine. Therefore, it was further purified from faeces. The mass spectra obtained from the urine metabolite had revealed that the morpholine ring of V-K 774 might have been hydrolized. M 8 was purified several times by TLC and after silylation its mass spectrum yielded a diethylamino substituent on the pyrimidine moiety (Table 2).

## DISCUSSION

The advantage of using drugs labelled with stable and radioactive isotopes is that: (i) only minute amounts of radioactivity are required for quantitative determination of elimination products: (ii) microgram quantities of impure metabolite suffice for running a mass spectrum which can be interpreted by simple differentiation of the artificially formed isotope clusters from other biological impurities. Therefore the time consuming purification of such a metabolite can be drastically reduced. This is evident, for example, in metabolites M 1, M 2 and M 3.

At present the use of radioactively labelled drugs is the most convenient approach for critical examination of pharmacokinetics and drug metabolism. $^{14}C$ was chosen in favour of $^{3}H$ because $^{3}H$ label is more susceptible to biodegradation than $^{14}C$, and random exchange of $^{3}H$ for H might cause substantial losses of radioactivity. Carbon isotopes can be chemically introduced into the main framework of a drug molecule and are therefore more resistant to biotransformation. In this way, most of the metabolites and the majority of their fragment ions in the corresponding mass spectra, are specifically characterized. This is demonstrated by the metabolites M 1 and M 7.

* The radioactive material at the origin is still unidentified.

Ultraviolet absorption, fluorescence, and colour reactions are commonly used for detecting drug metabolites during their isolation procedures. However, all these methods depend on the structure of the corresponding biotransformation products. In contrast, even very small amounts of radioactivity can be monitored and quantified, regardless of the structural characteristics of the metabolites. The metabolites can be more rapidly located on the TLC-plates using the spark chamber.

The advantages of double labelled drugs are also evident when employing other isolation techniques, e.g., gas–liquid chromatography and liquid chromatography.

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