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THIN-LAYER DENSITOMETRIC DETERMINATION OF MUZOLIMINE (BAY g 2821), A STRUCTURALLY NEW DIURETIC DRUG, AT THE NANOGRAM LEVEL IN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive and specific thin-layer densitometric method was developed for the determination of muzolimine (BAY g 2821), a structurally new diuretic drug of the pyrazolinone type. For detection of the drug on thin-layer chromatographic plates, a colour reaction with 4-dimethylaminocinnamaldehyde is carried out. The detection limit was 50 pg per spot on commercially available silica gel 60 plates. A nearly linear relationship between integrated peak area and amount per spot was obtained in the range from 100 pg to 1 μg per spot.

For quantitative determination in biological fluids, a recovery of about 100% is achieved by a single extraction with dichloromethane, and the extract is spotted directly on to the plate. The detection limit of muzolimine in plasma and urine was 1 ng/ml, and inaccuracy in the nanogram range was found to be 5–8%. The assay was applied to pharmacokinetic studies and can be recommended for monitoring plasma levels of muzolimine in patients.

INTRODUCTION

Muzolimine (BAY g 2821), 3-amino-1-(3,4-dichloro-α-methylbenzyl)-2-pyrazoline-2-one, is a new diuretic drug which differs structurally from other diuretics[1]. Investigations in dogs, normal subjects and patients have shown it to be a potent loop diuretic combining high ceiling activity with a thiazide-like prolonged duration of action[2–4]. In healthy volunteers, a single dose of 30 mg of BAY g 2821 was as effective as 40 mg of furosemide[5].

Prior attempts to detect the drug in blood plasma revealed that for pharmacokinetic studies an analytical method was required that is suitable for the quantitative determination at the nanogram level. Thin-layer densitometry was chosen because it is specific, rapid, easy to perform and no time-consuming clean-up steps are necessary. However, detection in the UV region and fluorescence quenching have been shown not to be sensitive enough. Therefore, a thin-layer densitometric assay based on the colour reaction of the amino group of muzolimine with 4-dimethylaminocinnamaldehyde was developed.

EXPERIMENTAL

Apparatus

A Zeiss PMQ II chromatogram spectrophotometer (Zeiss, Oberkochen, G.F.R.) was used, coupled to a potentiometric recorder (Servogor S Model RE 541, Metrawatt, Nürnberg, G.F.R.) and an electronic integrator (Minigrator, Spectra-Physics, Santa Clara, Calif., U.S.A.). Part of the study was carried out with a Schoeffel SD 3000 densitometer (Schoeffel, Westwood, N.J., U.S.A.) attached to a Philips PM 8000 recorder with a disc integrator. Figs. 1–7 are based on measurements with the Zeiss instrument.

TLC plates

Pre-coated silica gel 60 glass plates without fluorescent indicator, 20 × 20 cm, with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.) were used (referred to as "conventional plates" where necessary), and silica gel 60 HPTLC plates without fluorescent indicator, 10 × 10 cm (Merck). The plates were used without prior washing.

Reagents and solvents

All reagents and solvents were of guaranteed reagent grade and were used without further purification. 4-Dimethylaminocinnamaldehyde was obtained from Fluka (Buchs, Switzerland).

Standard solutions

A 10.00-mg amount of muzolimine, accurately weighed on a Mettler H20T analytical balance, was dissolved in 10.00 ml of dichloromethane (solution A, 1 μg/μl). The solution was diluted 1:10 with dichloromethane (solution B, 100 ng/μl), and so on to obtain solutions containing 10 ng, 1 ng, 100 pg and 10 pg of the drug per microlitre. Although muzolimine is stable in dichloromethane, standard solutions were prepared daily.

Spray reagent

A stock solution was prepared by dissolving 2.0 g of 4-dimethylaminocinnamaldehyde in 100 ml of 6 *M* hydrochloric acid and adding 100 ml of ethanol. The stock solution is stable for several weeks when stored in a refrigerator. The final spray reagent, which is stable at room temperature for several hours, was obtained by diluting 10 ml of the stock solution with 80 ml of ethanol.

Extraction

A 1-ml volume of blood plasma or urine was shaken for 10 min in a 10-ml glass-stoppered centrifuge tube with 500 μl of dichloromethane using a mechanical shaker (Minishaker, Braun, Melsungen, G.F.R.). The mixture was centrifuged for 10 min at 1200 *g*. With drug concentrations lower than 10 ng/ml, 2 ml of plasma or urine were extracted with 500 μl of dichloromethane.

Spotting the plates

Unknown samples were analysed in duplicate on two TLC plates. Aliquot volumes of the 500-μl extract, normally 5–100 μl for conventional plates and 1–20 μl

for HPTLC plates, were spotted using Hamilton syringes. Five to seven of the unknown samples were applied on to one plate, together with appropriate reference spots from stock solutions at the left- and right-hand sides of the unknowns. The centres of the spots were 12 mm apart on 20 × 20 cm conventional plates (15 spots) and 7 mm apart on 10 × 10 cm HPTLC plates (13 spots). When spotting volumes of more than 10 μl, care was taken in order to obtain small spots.

Development

Plates were developed in chloroform–ethanol (9:1), conventional plates to a height of 15 cm and HPTLC plates to a height of 6 cm, using a solvent-saturated, paper-lined rectangular tank.

Staining

After development, the plates were air dried and then sprayed uniformly with the spray reagent until the plates were transparent. After drying under a stream of cold air, the plates were heated in an oven for 7–8 min at 100°, yielding blue spots on a nearly white background. The plates were allowed to cool to room temperature and stored one on top of another, in order to prevent access of air.

Measurement and quantitation

Measurements on the plates were carried out in the reflectance mode at 610 nm in the direction of the solvent flow with a 3.5 × 0.1 mm slit, scanning speed 7.5 cm/min and paper speed 6 cm/min. If the volumes of the unknown samples spotted on conventional TLC plates were greater than 100 μl, it was sometimes necessary to use a 6 × 0.1 mm slit.

Calculation of the concentrations in the unknown samples was achieved from calibration graphs constructed for each plate by plotting peak areas (integrator counts) *versus* amount of drug in the reference spots. The recorder was only used for adjusting the spot to be measured under the slit of the densitometer, and to record the peaks to obtain a densitogram for documentation.

Recovery experiments

Blank plasma and urine were spiked with known amounts (5 ng/ml–2 μg/ml) of the drug dissolved in ethanol and analysed as described above. During and after pharmacokinetic investigations, spiked samples for recovery control were analysed, the actual concentrations being unknown to the operators.

RESULTS AND DISCUSSION

Stability in biological fluids

Muzolimine was found to be unstable in polar solvents and in biological fluids even when stored deep-frozen at −30°. Therefore, ethanolic solutions for spiking blank plasma and urine were used immediately after preparation.

A systematic study, however, revealed that stabilization of muzolimine in plasma and urine is achieved by addition of 1 mg/ml of L-cysteine hydrochloride (Merck). Thus, muzolimine was stable in biological fluids for 12 h at room temperature and for at least 96 h at 8° (refrigerator). No decrease in drug concentration was

found in plasma and urine stabilized with cysteine hydrochloride and stored deep-frozen for several weeks, and no interference in the assay was observed.

Detection limit

The lowest detectable amount of muzolimine was 50 pg per spot on HPTLC plates and 100 pg per spot on conventional plates. The recorded peaks are clearly recognizable compared with the background scan, an example being given in Fig. 1 with repeated scans of 50, 100 and 250 pg per spot on an HPTLC plate.

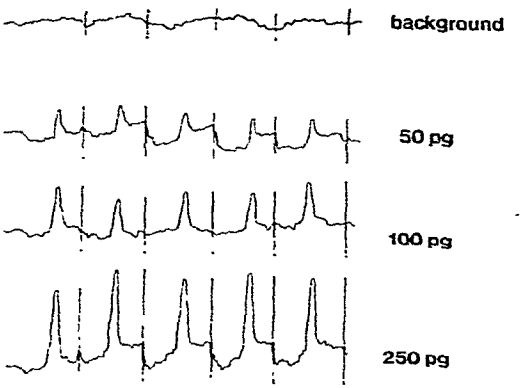


Fig. 1. Repeated scans of muzolimine spots in the picogram range after staining (HPTLC plate, Zeiss chromatogram spectrophotometer).

Under the conditions described under Experimental, the instrument can reliably integrate an area under the peak of 50 units, corresponding to 100 pg per spot. Therefore, it was calculated that the detection limit of muzolimine in biological fluids should be 250 pg/ml when spotting 200 μl of a 500-μl extract on conventional plates. However, this level could not be achieved in practice, and the detection limit was found to be 1 ng/ml in both plasma and urine.

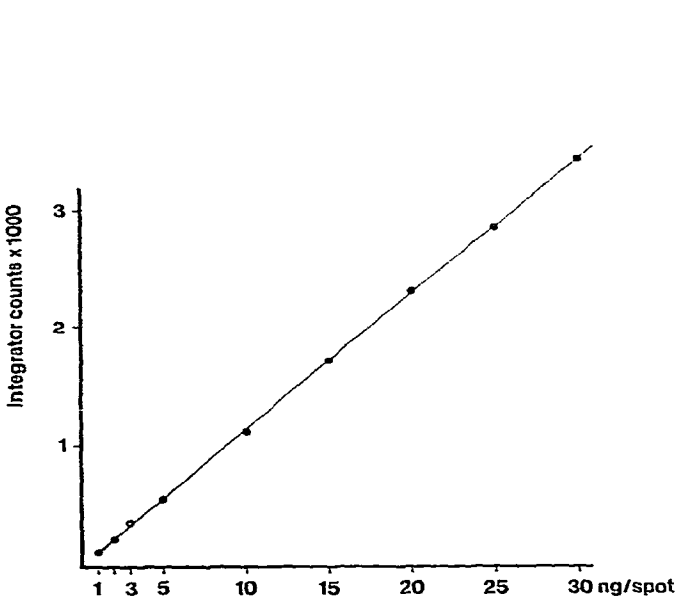
Spotted volume and spot diameter

Is is often claimed that in quantitative *in situ* thin-layer chromatography different amounts of a substance should be spotted in equal volumes, in order to obtain a high accuracy. However, in pharmacokinetic studies we have to deal with biological material of variable composition. There are not only species differences but also interindividual differences within one species, and even differences between samples from one individual, for example before and after a meal. The accuracy of an assay is influenced much more by these differences than by the inaccuracy of spotting different volumes. In addition, even when the standards are applied in volumes of 1–10 μl, this is not possible with the extracts from biological material, except when the drug levels are very high and the detection limit is very low.

During the development of the assay for muzolimine, we tried, therefore, to achieve small spots by improving the technique of spotting and by studying the properties of several solvents. It was found that dichloromethane is most suitable, and that there is nearly no difference between the areas under the peaks of spots

applied with 1 and 10 μl, or 5 and 50 μl. This may be also due to the physico-chemical properties of muzolimine, *i.e.*, the drug seems to be absorbed during spotting by silica gel on a very small area even when the solvent spot is larger.

This is demonstrated by the straight calibration line in Fig. 2, where the amounts were applied as spots in volumes of 1–10 μl. The peaks shown in Fig. 3 were obtained by scanning 5-ng spots applied on to the plate in volumes of 5 (standard solution), 25 and 150 μl (plasma extracts, recovery about 100%). The shape of the peaks is not significantly influenced by the spotted volume.



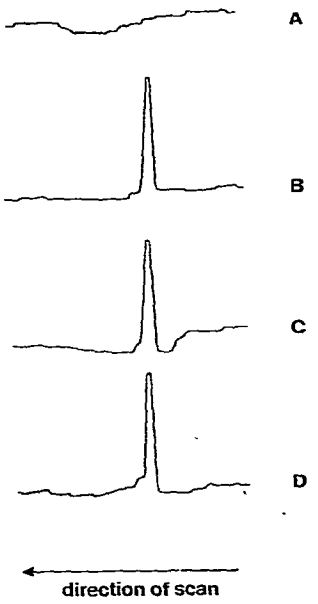


Fig. 2. Calibration line of muzolimine in the low nanogram range.

Fig. 3. Typical peaks observed when scanning a TLC plate with extracts from plasma (1 ml of plasma extracted with 500 μl of dichloromethane, part of which is applied as a spot). A, Extract of blank human plasma, 25 μl spotted; B, reference spot of 5 ng of muzolimine (spotted volume 5 μl); C, extract of 1 ml plasma spiked with 100 ng of muzolimine (spotted volume 25 μl, corresponding to 0.05 ml of plasma); and D, spiked plasma with a concentration of 20 ng/ml (spotted volume 150 μl, corresponding to 0.3 ml of plasma). The shape of the peaks is not significantly influenced by the spotted volume.

Calibration graphs

A nearly linear relationship between the integrated peak area and the amount of muzolimine on the plate was observed between 100 pg and 1 μg per spot. A typical calibration line is shown in Fig. 2, and a densitogram in Fig. 4. Similar calibration lines were obtained between 100 pg and 5 ng, or 100 ng and 1 μg per spot. When the range of amount per spot on one plate is greater than 1:50, as is the case when analysing unknown samples, slightly curved lines were obtained, as shown in Fig. 5.

Stability of coloured spots

A crucial parameter in thin-layer densitometry of spots made visible by

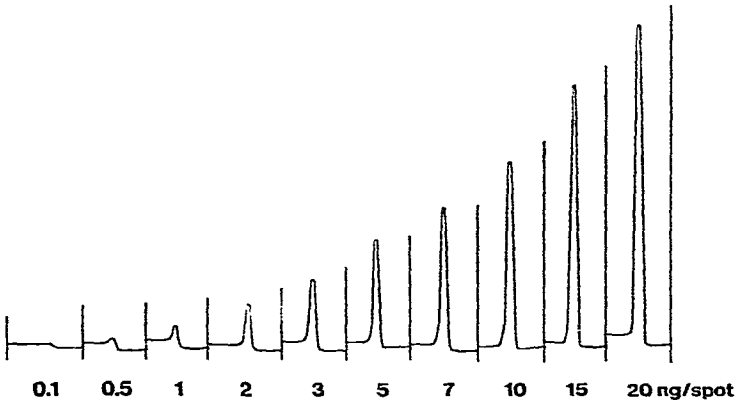


Fig. 4. Densitogram of a TLC plate with muzolimine standards in the low nanogram range.

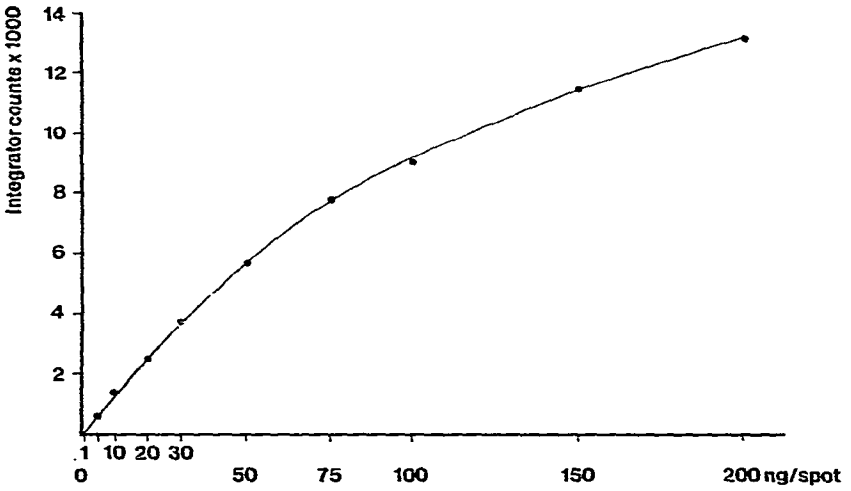


Fig. 5. Muzolimine calibration graph covering the range from 1 to 200 ng per spot.

staining is the stability of the colour[6]. Repeated measurements of muzolimine spots after reaction with 4-dimethylaminocinnamaldehyde, yielding the corresponding Schiff base, did not indicate a decrease in response during 10 scans. However, plates with reference spots scanned at different intervals up to 11 days after staining revealed a change in response. As a result of these experiments, it was concluded that measurements are preferably carried out 16–20 h after staining, because this represents the maximum response (Fig. 6). Later the response declines slightly and then remains more or less unchanged for several days. During this time the background of the plate, which is nearly white at the beginning, becomes pink and finally red, but this change in the background does not influence the measurement of the blue spots at 610 nm. The high stability of the coloured spots is of particular advantage in routine assay.

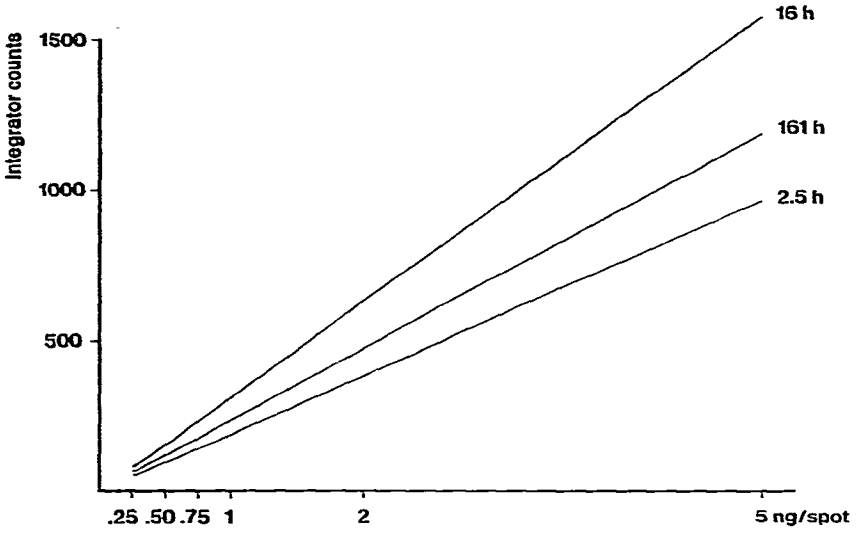


Fig. 6. Calibration lines for a TLC plate obtained by measurement at different intervals after staining.

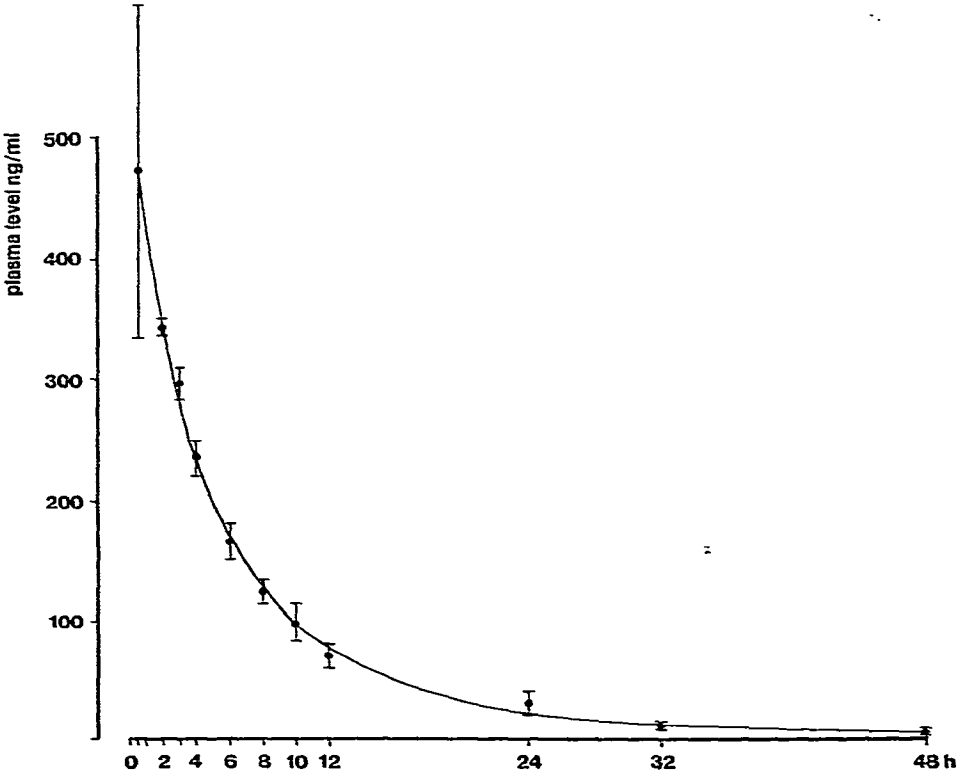


Fig. 7. Mean muzolimine plasma levels (± S.D.) of three volunteers after ingestion of 30 mg of muzolimine as tablets (thin-layer densitometric assay).

Specificity

So far more than 500 plasma and urine samples from animals, healthy volunteers and patients have been analysed, and no interfering compounds have been found, even in samples from patients undergoing multi-drug therapy. It seems, therefore, that the method described is specific for muzolimine ($R_F = 0.21$), due to extraction, chromatography and the colour reaction. However, no systematic study has yet been carried out on drugs concomitantly administered to patients with chronic heart failure, liver disease and renal insufficiency.

Recovery

In the concentration range from 40 to 2000 ng/ml a recovery of about 100% is achieved by a single extraction step (Table I). At lower concentrations the recovery is about 85%, but this is probably the result of spotting 200-μl volumes of the dichloromethane extract, which is necessary with samples in the low nanogram range. If care is taken to spot higher volumes very slowly, the peak area is not significantly influenced by volumes up to 150 μl (Fig. 3). However, during routine assay volumes of 150–250 μl are often applied too fast, resulting in spots, the areas of which are 15–20% smaller when compared with the same amounts spotted with smaller volumes.

Recovery is subject to the individual performance of the assay, and is therefore controlled during pharmacokinetic studies by each operator involved and at the actual concentrations of the biological samples. Results obtained upon measurement are, if necessary, corrected for loss of recovery.

TABLE I

RECOVERY OF MUZOLIMINE FROM PLASMA AND URINE

Sample	*Amount added (ng/ml)*	*Number of samples*	*Mean recovery (%)*	*Relative standard deviation*
Plasma	250–1250	5	101.3	4.33
	170– 580	7	93.8	4.52
	50– 500	8	108.2	2.99
	300 and 400	5	98.6	7.35
	200 and 500	5	94.2	5.28
	100	5	106.9	3.40
Urine	500–2000	9	99.7	3.62
	1000	10	97.4	4.56
	40– 500	11	101.9	4.51

Precision and accuracy

Precision was studied for single steps of the method. The relative standard deviation of 10 scans of one spot in the range from 30 to 500 ng per spot was $< 0.5\%$, and was about 3% for repetitive manual spotting of volumes of 5–25 μl with amounts of 10–500 ng per spot. The precision is also demonstrated by the calibration graphs, typical examples being shown in Figs. 2 and 5.

The accuracy of the whole assay was investigated in a large number of recovery studies carried out under conditions of routine assay (Table I). An example in which

spiked plasma samples simulating plasma levels after a single dose of 30 mg of muzolimine were analysed by three operators is shown in Table II. Overall, inaccuracy in the concentration range from 30 to 2000 ng/ml was 5–8%, which is acceptable in pharmacokinetic studies. At lower concentrations (2–20 ng/ml) inaccuracy was found to be 10–15%.

TABLE II

ACCURACY OF DETERMINATION OF MUZOLIMINE IN PLASMA

The spiked samples were analysed in duplicate by three operators (A, B and C) and corrected for loss of recovery.

Muzolimine added (ng/ml)	*Muzolimine found (ng/ml)*		
	A	*B*	*C*
357	342	341	381
420	429	426	460
294	308	308	321
252	263	278	255
200	205	198	193
168	153	149	158
116	110	123	108
84	85	85	78
59	56	56	54
32	33	30	—

Conventional versus HPTLC plates

For the determination of muzolimine after extraction from biological material, HPTLC plates offer no particular advantage over conventional plates. Using the latter, 12–15 samples can be analysed in duplicate during an 8-h day, including construction of the calibration graphs and calculation of the unknown concentrations. Although the development of HPTLC plates is completed after 6 min (compared with conventional plates after 20 min), the number of samples analysed per day could not be increased. The most time-consuming step is spotting the plates, which is much easier on conventional plates than on HPTLC plates when volumes of more than 10 μl have to be spotted. However, small spots were even obtained on HPTLC plates after spotting up to 150 μl of an extract from plasma, but this requires much more time compared with conventional plates. On the other hand, spotting nanolitre volumes on HPTLC plates would result in restriction of the assay to samples with high muzolimine concentrations.

Muzolimine plasma levels

Plasma levels of muzolimine were studied in mice, rats, dogs, normal subjects and patients[7]. Prior studies were carried out by extracting the samples twice with diisopropyl ether, reducing the organic phase under a stream of nitrogen to a volume of 300 μl and spotting an aliquot on to the plate. The method described here is an improvement inasmuch as only one extraction step is required and part of the organic layer is spotted directly. This involves considerably less time, and the accuracy of the overall assay is increased.

The applicability of the method to pharmacokinetic investigations is demonstrated by the mean plasma level curve for three healthy volunteers after ingestion of 30 mg of muzolimine (Fig. 7). Concentrations of the unchanged drug were determined up to 48 h after administration and declined with a half-life of 3.5 h during the first phase of disappearance from plasma. The decline of the plasma levels is paralleled by the pharmacodynamic action of the drug[8].

CONCLUSION

A thin-layer densitometric method has been developed for muzolimine (BAY g 2821), based on a colour reaction with 4-dimethylaminocinnamaldehyde at 100°. The detection limit of the pure substance is 50 pg per spot on HPTLC plates, and 100 pg per spot on conventional plates.

For quantitative determination of muzolimine in biological fluids only a single extraction is required, and an aliquot of the extract is spotted directly on to the TLC plate. The proposed method, with a detection limit of 1 ng/ml, is specific, rapid and accurate, thus providing an easily performed routine assay in the nanogram range. By modification of the extraction procedure, the assay could probably be extended to the quantitative analysis of picogram amounts of muzolimine in biological fluids.

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