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DETERMINATION OF NANOGRAM AMOUNTS OF PRIMARY AROMATIC AMINES AND NITRO COMPOUNDS IN BLOOD AND PLASMA

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SUMMARY

A rapid and highly sensitive method, based on the direct fluorimetric scanning of thin-layer chromatograms, is described for the quantitative determination of flunitrazepam and its main metabolites in human blood or plasma. This method is generally applicable to aromatic nitro compounds that are reducible with tin(II) chloride. In particular, it is possible to determine primary amines in nanogram amounts. Fluorescamine is used as a reagent to produce fluorescent derivatives. The method is suitable for pharmacokinetic studies of flunitrazepam and its main metabolites in human blood and plasma.

INTRODUCTION

For pharmacokinetic studies on flunitrazepam*, an assay procedure for the unchanged substance and its main metabolites in body fluids was required. The structures of the drug and its main products of biotransformation are shown below.

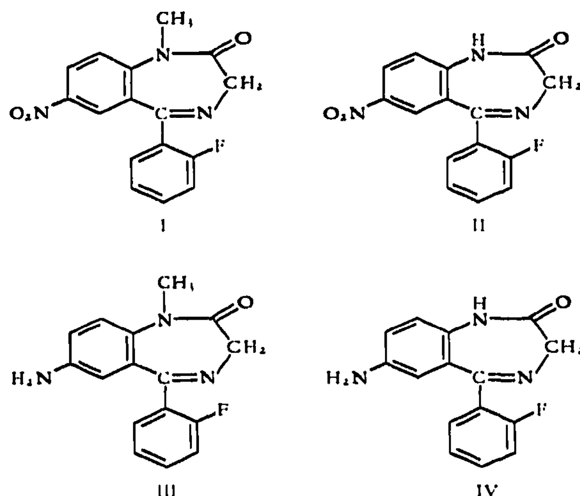
There are methods for the determination of flunitrazepam (I) and its 7-nitrodesmethyl metabolite (II) by gas chromatography[1,2], but the 7-amino (III) and the 7-aminodesmethyl metabolites (IV) cannot be detected by this technique in the concentrations that occur in human blood after therapeutic doses.

We therefore developed a thin-layer chromatographic (TLC) method suitable for the determination of flunitrazepam and its main metabolites, also including the amino compounds. The general principle of the method is applicable to other aromatic nitro compounds and also to aromatic and aliphatic primary amines. The procedure is rapid and not difficult to perform. The sensitivity of the method lies in the region of 1–2 ng/ml in blood or plasma.

PRINCIPLE OF THE METHOD

Flunitrazepam and its main metabolites are extracted from human plasma or

* Active principle of the hypnotic preparation Rohypnol (Roche trade mark).



blood into methylene chloride at a pH of 8.5–9.0. The organic phase is concentrated and applied to a thin-layer silica gel plate. After development, the nitro compounds on the thin-layer plate are reduced with a solution of tin(II) chloride in dilute acetic acid. The plate is then buffered to a pH of 8.4, sprayed with a solution of fluorescamine and the fluorescence of the spots directly quantitated with a suitable scanner, the levels being calculated by comparison with standards added to drug-free plasma or blood.

EXPERIMENTAL

Reagents

Buffer of pH 9. Dissolve 23.5 g of potassium chloride, 19.75 g of sodium carbonate and 19.5 g of boric acid in 800 ml of distilled water, adjust the pH to 9.0 and make the volume up to 1 l with distilled water.

Buffer of pH 8.4. Dissolve 31 g of boric acid in 100 ml of 1 *N* sodium hydroxide solution and add 800 ml of distilled water. Adjust the pH to 8.4 and make the volume up to 1 l with distilled water.

Tin(II) chloride solution. Dissolve 4 g of tin(II) chloride dihydrate in 100 ml of 5% acetic acid and add 1 ml of a 0.5% solution of phenolphthalein in dioxane.

Sodium hydroxide solution. Prepare a 2% solution of sodium hydroxide in distilled water.

Methylene chloride solution for extraction. Add 1 ml of analytical-reagent grade toluene to 100 ml of analytical-reagent grade methylene chloride.

Fluorescamine solution. Dissolve 100 mg of fluorescamine (Fluram) in 100 ml of analytical-reagent grade acetone.

Solvents for thin-layer chromatography. The following analytical-reagent grade solvents are used: methanol, benzene, methyl acetate, chloroform, *n*-hexane, toluene and methylene chloride.

Standards

Stock solutions. Dissolve 2 mg of flunitrazepam in 20 ml of methylene chloride in a calibrated flask. Prepare corresponding solutions with 2 mg of each of the three main metabolites II, III and IV.

Standard solution for thin-layer chromatography. It is advantageous to use as the standard a mixture of flunitrazepam and its main metabolites. Place 1 ml of each of the above stock solutions in a 20-ml calibrated flask and make up to the mark with methylene chloride. Dilute 1 ml of this solution to 20 ml with methylene chloride; 10 μl of the resulting solution contain 2.5 ng of flunitrazepam and 2.5 ng of each of the main metabolites.

Plasma standard solutions. Place 1 ml of each of the above stock solutions in a 20-ml calibrated flask and make up to the mark with methanol. Dilute 5 ml of this solution to 25 ml with methanol. Place 0.2 ml of the resulting solution in a 20-ml calibrated flask and make up to volume with plasma containing no interfering substances (this must be checked very carefully); 1 ml of this first plasma standard contains 10 ng of flunitrazepam and of each of the main metabolites. Then 5 ml of this plasma standard is diluted to 10 ml with the same plasma; this second standard contains 5 ng/ml of flunitrazepam and of each of the main metabolites.

Materials for thin-layer chromatography

Merck (Darmstadt, G.F.R.) silica gel 60 F_{254} 0.25-mm pre-coated thin-layer plates were used. The usual 10-μl micropipettes can be used, *e.g.*, those of Dr. Barrolier.

The chromatographic tank is shown in Fig. 1. A suitable apparatus for continuous-elution TLC after Truter[3] is available from Shandon Southern Instruments.

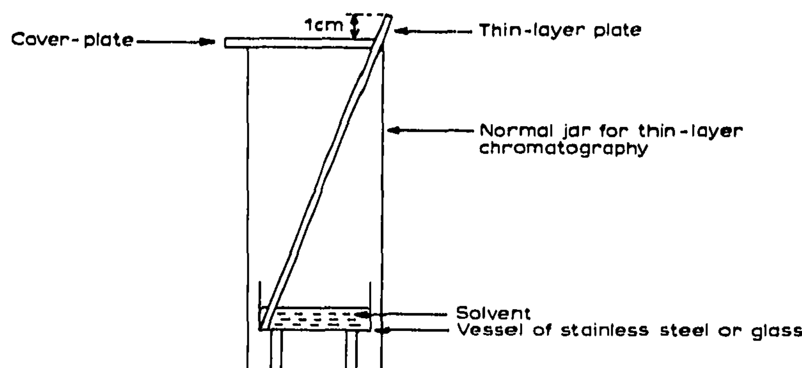


Fig. 1. Jar for continuous development of thin-layer plates. Three sides of the jar are lined with filter-paper in order to achieve vapour saturation rapidly.

There are several scanners for TLC that are suitable for measuring the fluorescence of the spots (excitation wavelength 405 nm, fluorescence wavelength 495 nm), but the sensitivity limit depends strongly on the quality of the instrument. We used a modified Aminco thin-film chromatograph scanner.

Procedure

Extraction. Measure into 15-ml stoppered centrifuge tubes 1 ml of the plasma to be analyzed or the plasma standards containing 10 or 5 ng/ml of the compounds in question, 0.5 ml of buffer of pH 9, and 5 ml of methylene chloride extraction solution (in the case of blood, add only 0.2 ml of buffer of pH 9). Stopper the tubes and, in order to avoid emulsion formation, rotate them mechanically around the horizontal axis for 10 min (*ca.* 60 times per minute). Centrifuge the tubes for 10 min at 700 *g*. Aspirate off as much as possible of the upper phase and measure 4 ml of the organic phase into a new 10–15-ml tube. Place the tubes in a shaking thermostat at 55° and shake them until most of the lower-boiling solvent has evaporated and the higher-boiling toluene remains (about 40–50 μl); this usually takes 10–15 min.

Application to the thin-layer plate. The spots are applied 2 cm from the edges of the plate, the distance between the spots being 1.5 cm. In order to obtain small spots, which is very important for ensuring a high sensitivity, the starting zone of the thin-layer plate is heated to about 60° with a heating plate. The residue from the extraction is transferred quantitatively to the plate with a 10-μl micropipette. The tube is rinsed with 50 μl of methylene chloride and the rinsings are also applied to the plate.

Place on the same plate 20 and 40 μl of the *Standard solution for thin-layer chromatography* in portions of 10 μl (*i.e.*, 5 ng and 10 ng of flunitrazepam and its main metabolites, respectively).

Chromatography. The thin-layer plate is placed in the jar designed for continuous thin-layer chromatography. The jar is lined with filter-paper so as to achieve vapour saturation. It is recommended that the plate should not be developed for more than 12–15 cm (the time required for this is usually about 45 min). Suitable solvents and approximative R_F values are listed in Table I. It should be mentioned that a jar for continuous TLC is used but that the principle of our procedure corresponds to normal TLC with saturation of the jar. Thus we can estimate R_F values. In our experience, this is the simplest way to separate interfering plasma components from the substances to be determined.

Detection. After development, dry the plate and spray it with the tin(II) chloride solution until the layer is uniformly wet. Dry the plate for 10 min at 105–110° and, after cooling, spray it first with 2% sodium hydroxide solution until a faint purple colour appears and then with buffer of pH 8.4; again, the plate must be completely

TABLE I

SOLVENTS FOR THIN-LAYER CHROMATOGRAPHY OF FLUNITRAZEPAM AND ITS MAIN METABOLITES

Solvent	*Approximate R_F values of the substances*			
	I	*II*	*III*	*IV*
Benzene–acetone (10:6)	0.5	0.4	0.3	0.15
Methyl acetate–chloroform (1:1)	0.35	0.25	0.15	0.1
Methyl acetate–chloroform (2:1)	0.45	0.35	0.25	0.15
Methyl acetate–toluene–*n*-hexane (2:1:1)	0.35	0.3	0.15	0.08
Methyl acetate–toluene–methylene chloride (2:1:1)	0.35	0.25	0.15	0.1

wet. Wait until all of the solution has penetrated into the layer and then spray it with 0.2% fluorescamine solution. It is important to vaporize the solution carefully; always use an excess of reagent (except for 2% sodium hydroxide solution). Dry the chromatogram with a stream of warm air (about 70°) and examine it under longwave UV light (366 nm). Amounts of 5 and 10 ng of the substances become visible with yellow fluorescence.

Scanning. Measure the fluorescence of the spots at the excitation wavelength (405 nm) and the fluorescence wavelength (495 nm), and record the curves.

Calculation. As the height of the signals is directly proportional to the amount applied to the plate, measure the heights of the signals for the 5 and 10 ng/ml plasma standards, calculate the mean height equivalent to 1 ng/ml and compare this mean value with the heights of the signals for the samples being analyzed. Then,

$$\frac{H_S}{H_{PS}} = \text{concentration of substance in plasma (ng/ml)}$$

where H_S = height of signal for the sample and H_{PS} = height of signal for 5 ng/ml plasma standard plus height of signal for 10 ng/ml plasma standard divided by 15, *i.e.*, mean height corresponding to a concentration of 1 ng/ml in plasma.

RESULTS AND DISCUSSION

The conditions used in the procedure described (concentrations of reagents, temperature, time) were established from many experiments and depending on the substances being investigated, they can be modified. The reproducibility of the method depends mainly on the uniformity of vaporization, which must be as homogeneous as possible.

In order to check for interfering substances in the plasma, it is advantageous to use two or more solvents for the development of the thin-layer chromatograms.

By comparison of the fluorescence of the directly applied substances with the fluorescence of the substances added to plasma, it is possible to determine the overall yield of the procedure. Taking into account the aliquot loss due to proceeding with only four-fifths of the methylene chloride extract, the yield lies between 70 and 90%, depending on the substances. The extraction yield is higher, but as there are components of the plasma, which do not fluoresce but show the same migration as the substances being determined, the fluorescence of the latter is decreased. These interfering components are probably lipids.

Linearity

In the region of 1–20 ng of flunitrazepam and its metabolites, the height of the fluorescence signals is directly proportional to the amounts on the thin-layer plate.

Sensitivity limit

The sensitivity limit depends on the substance being examined. It is interesting that the desmethyl metabolites (II and IV) of flunitrazepam show only half of the fluorescence intensities of the corresponding N-methyl compounds (I and III).

In general, the following concentrations can be measured: substances I and III, 1 ng/ml; substances II and IV, 2 ng/ml in blood or plasma. With special care, half of these concentrations can be detected. The sensitivity for the pure substances is about 0.5 ng per spot.

Reproducibility

Two technicians analyzed the same plasma samples on different days. The results are given in Table II. In this instance, the concentrations of II and IV were below the detection limit.

TABLE II

PLASMA LEVELS (ng/ml) OF FLUNITRAZEPAM AND METABOLITE III AFTER A SINGLE ORAL DOSE OF 2 mg OF FLUNITRAZEPAM, AS DETERMINED INDEPENDENTLY BY TWO TECHNICIANS

The concentrations of metabolites II and IV in the plasma were below the detection limit of 2 ng/ml. A = results of technician A; B = results of technician B.

Patient No.	*Sample No.*	*Time after medication (h)*	*Substance I*			*Substance III*		
			A	*B*	*Mean*	*A*	*B*	*Mean*
1	101	½	12	12.5	12.3	1.5	2	1.8
	102	1	10.5	10	10.3	3	3	3
	103	2	10.5	9.5	10	4	3	3.5
	104	4	9	8	9.5	4	4	4
	105	8	7	7.5	7.3	4	4	4
	106	12	5.5	4.5	5	4.5	5.5	5
	107	24	3.5	4	3.8	5	4.5	4.8
	108	48	2	2	2	2.5	3.5	3
2	201	½	9.5	8.5	9	1	1.5	1.3
	202	1	14	14.5	14.3	1.5	1.5	1.5
	203	2	16	16.5	16.3	2.5	2	2.3
	204	4	12.5	12	12.3	3	3.5	3.3
	205	8	8	6.5	7.3	4	3	3.5
	206	12	6.5	6.5	6.5	3.5	3	3.3
	207	24	4.5	4	4.3	2.5	3.5	3
	208	48	2	2.5	2.3	2.5	3	2.8

The standard deviations of the determinations were ± 0.52 ng/ml for substance I and ± 0.46 ng/ml for substance II. For the mean of two determinations, the standard deviation (s) is therefore

$$s_{\text{mean}} = \frac{s}{\sqrt{2}}$$

and the values of s_{mean} were ± 0.37 ng/ml for substance I and ± 0.33 ng/ml for substance II.

The utility of the procedure is illustrated in Fig. 2, which shows the plasma level curve of flunitrazepam for one patient (No. 2) with the corresponding standard deviation.

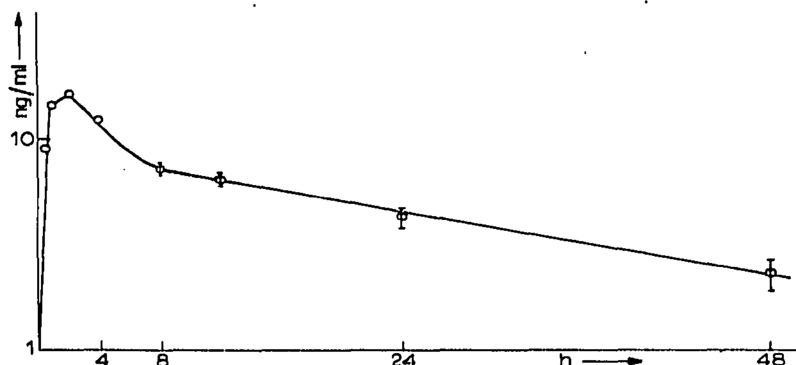


Fig. 2. Plasma levels of unchanged flunitrazepam after a single oral dose of 2 mg by one patient (No. 2).

Specificity

The normal components of human plasma or blood do not usually interfere. However, in one study, all of six volunteers showed the same interfering substance after a common meal, which probably originated from the flesh of animals that had been medicated shortly before slaughtering.

Out of about 20 patients treated with high concentrations of other drugs, in only one instance could flunitrazepam and its metabolites not be determined because of an interfering peak.

Four volunteers received flunitrazepam labelled with carbon-14. The plasma samples were analyzed by TLC combined with scintillation counting, and the same samples were also analyzed by the described fluorimetric method. Both procedures gave identical results within the standard deviation of the methods, which indicates that the fluorimetric determination has a high specificity.

Practicability

Prior to its use for actual investigations, it is necessary to practise the method with pure substances and standards added to plasma or blood until the results are reproducible. Once this has been achieved, the method presents no problems. The main difficulty may come from interfering compounds present in food. A well trained technician is capable of analyzing, with ease, 20–30 samples of plasma or blood per day.

The only apparatus that is usually not readily available in a clinical laboratory is a scanner for the fluorimetric evaluation of the thin-layer plates. Nevertheless, we consider that in the future, densitometric methods for examining thin-layer plates will provide a very powerful means of determining the blood and plasma levels of drugs and their metabolites, which form the basis of pharmacokinetic investigations.

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