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Determination of heptaminol in plasma by thin-layer chromatography and *in situ* fluorimetry

A. MORROS†, L. BORJA and J. SEGURA*

Division of Biochemistry and Biopharmaceutics, Institute of Research, Laboratorios Almirall S.A., Cardoner, no 68–74, Barcelona-24, Spain

Abstract: A method has been developed for the determination of heptaminol in plasma samples. The main steps involved in the assay are: ethereal extraction at an alkaline pH; concentration of the extracted heptaminol as its hydrochloride; formation of a fluorescent derivative by reaction with 4-chloro-7-nitrobenzo-2,1,3-oxadiazole; separation of the fluorophore by thin-layer chromatography and *in situ* quantitation of the emitted fluorescence by photodensitometry. The sensitivity limit is estimated to be 25 ng ml^{-1} plasma, the overall recovery is 81% and the relative standard deviation is 6% at 200 ng ml^{-1}. The assay is useful in studies on the pharmacokinetics of heptaminol in animals or man after administration of therapeutic doses. Plasma levels of the drug following administration of 5 $mg.kg^{-1}$ to rats are reported.

Keywords: *Thin-layer chromatography; heptaminol;* in situ *fluorimetry; blood plasma analysis.*

Introduction

Heptaminol (6-amino-2-methyl-2-heptanol) is an adrenergic drug that produces vasodilation of coronary arteries and promotes the contractibility of the myocardium. Despite its use for many years in cardiovascular therapy, few pharmocokinetic data on the drug are available. Its urinary excretion has been studied [1–4], but its profile in the systemic circulation has been investigated only by the use of ^{14}C-labelled heptaminol [3, 4]. Plasma levels of 40–2000 ng ml^{-1} of radioactive material were measured after therapeutic doses (100 mg) by intravenous injection. A sufficiently sensitive and specific analytical method that does not involve the use of a radioactive tracer is needed to determine the drug in blood.

Gas–liquid chromatography was used by Rabiant *et al.* [1, 2] to determine heptaminol in urine. The low sensitivity reported (1 μg on column) makes this method unsuitable for analysis of plasma samples. According to other reports the formation of fluorescent

* To whom correspondence should be addressed.
† Present address: Department of Biophysics, Faculty of Medicine, Autonomous University of Barcelona, Bellaterra (Barcelona), Spain.

derivatives gives lower detection limits. Messerschmidt [5] obtained the dansyl derivative of heptaminol which can be detected in amounts as low as 300 ng by thin-layer chromatography. A more sensitive and specific reaction for aliphatic amines is condensation with 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD-Cl) described by Ghosh and Whitehouse [6]. Accordingly, Van Hoof and Heyndrickx [7] proposed a method to determine the concentration of heptaminol and other sympathomimetic drugs in plasma using the reaction with NBD-Cl to form derivatives which were quantified after separation by thin-layer chromatography. Although the method was useful for amphetamine, its applicability to heptaminol was impaired by the very low extraction recovery from plasma and by interference on the chromatograms.

To attain a lower detection limit for heptaminol in plasma a method has been developed for quantitative extraction of heptaminol and thin-layer separation of the heptaminol-NBD derivative without interference. The quantitation has been carried out by *in situ* photodensitometry of the emitted fluorescence [7]. The structure of the fluorophore has also been established.

Experimental

Materials

Stock solutions of heptaminol (free base) (Fabriques de Loire, Issy les Moulineaux, France) were prepared in distilled water in concentrations of 10–700 ng ml^{-1}. A 1% stock solution of 4-chloro-7-nitrobenzo-2,1,3-oxadiazole (NBD-Cl) (Aldrich Europe, Beerse, Belgium) was prepared in methyl isobutyl ketone (MIBK) and stored at 4°C. Diethyl ether saturated with hydrochloric acid was prepared immediately before use.

The test tubes used for evaporation of the ether extracts were silanized by treatment with a 5% solution of trimethylchlorosilane (Applied Sciences Laboratories Inc., State College, USA) in toluene for 18 hr. The tubes were then rinsed thoroughly with methanol and dried in an oven at 70°C.

The TLC plates were precoated with silica gel 60F 254 of 0·25-mm thickness (Merck, Darmstadt, FRG). All solvents and reagents were of analytical grade (Merck).

The plates were scanned with a Vitatron photodensitometer (model TLD 100) in the fluorescence mode. Excitation was carried out by means of a Hg lamp. A Vitatron recorder (model UR 400) was used. Identification of the structure of the isolated fluorophore was carried out by means of the following instruments: Hitachi–Perkin–Elmer RMU-6H (mass spectra), Perkin–Elmer R-24 (nuclear magnetic resonance spectra), Perkin–Elmer 207 (infrared spectra), Aminco–Bowman (fluorescence spectra) and Perkin–Elmer 240 (elemental analysis).

Biological samples

Preliminary tests of the method were done on human and rat plasma with added known amounts of heptaminol. To test the usefulness of the method a pharmacokinetic experiment was done with Wistar male albino rats (220–280g). Before the experiment all animals were fasted overnight. Doses of 5.0 mg kg^{-1} (8 ml of an aqueous solution kg^{-1}) were administered through a stomach tube to the rats which were then killed in groups of three at 15, 30, 45 min and 1, 1.5, 2, 3, 4 and 6 hr after administration. Blood was collected from the jugular vein into heparinized tubes and plasma was separated by centrifugation and kept at −20°C. Plasma was also obtained from nontreated rats.

Analytical procedure

Extraction from plasma. A 0.1 ml sample of 2 N NaOH was added to 0.5–1.0 ml of plasma and the mixture was extracted with 5.0 ml of diethyl ether in stoppered 10 ml tubes shaken for 1 hr. The phases were then separated by centrifugation at 2000 rpm for 10 min. Extracts were obtained in the same way from blank plasma and from spiked samples prepared by adding small volumes (10–100 μl) of a stock solution of heptaminol to 1 ml aliquots of blank plasma. The ethereal phases were transferred to silanized 10 ml tubes to which one drop (about 15 μl) of HCl-saturated diethyl ether was added to lower the pH below 8.0. The ether and the excess of HCl were then evaporated in a stream of nitrogen using gentle heat for 30 min.

Chemical reaction with NBD-Cl. A 0.2 ml portion of 0.1 M $NaHCO_3$ was added to each tube containing the dry residue of the etheral phase. The pH of the solution was then measured (approximately pH 9.0). A 0.2 ml portion of a solution of NBD-Cl (10 mg ml^{-1}) in methyl isobutyl ketone (MIBK) was added and the tubes were stoppered and heated at 80°C for 30 min. The tubes were cooled, 100 μl of the upper phase (MIBK) was transferred to glass tubes and the solvent was evaporated under a stream of nitrogen by heating at 80°C.

Measurement of fluorescence. The dry residues were taken up in chloroform and the solutions were applied to the thin-layer plates. The chromatograms were developed for 60 min in chloroform–methanol (90:10, v/v). The plates were dried at room temperature and stored in the dark until observation. The spots were viewed under a UV lamp fitted with a 366 nm filter.

The intensity of fluorescence emitted by the heptaminol derivative (R_F 0.45) was determined by photodensitometry in a dark room. Excitation light was selected using a 450 nm primary filter. A 0.50 mm slit slide was inserted between the chromatographic plate and the photomultiplier. The emitted fluorescence intensity was measured through a 525 nm secondary filter and continuously recorded. The areas of the peaks were calculated by triangulation. Concentrations of heptaminol in plasma were calculated by interpolation from a calibration curve.

Large scale preparation and isolation of the reaction product

To identify the precise structure of the fluorescent derivative a preparative scale experiment was carried out. A 100 ml sample of a solution of 1% NBD-Cl in MIBK was added to 100 ml of a slightly alkaline solution (0.1 M $NaHCO_3$) which contained 730 mg of the drug and the products were allowed to react under the conditions described above. After the reaction, the MIBK phase was evaporated and the dry residue redissolved in ethanol. Excess reagent and some by-products of the reaction were precipitated by addition of 4 volumes of distilled water. The heptaminol derivative was obtained by extraction with chloroform and was purified by adsorption chromatography on a 300 × 15 mm i.d. column packed with silica gel 60 (Merck, Germany) using chloroform-tetrahydrofuran (9:2, v/v) as eluent. Fractions of 2.0 ml were collected and aliquots of these fractions were analysed by thin-layer chromatography. The heptaminol derivative was recovered from the most pure fraction by evaporation. After dissolution in chloroform it was precipitated by addition of carbon tetrachloride. The dried powder was used for elemental analysis and for examination of mass, nuclear magnetic resonance, infrared and fluorescence spectra.

Results

Figure 1 shows a characteristic thin-layer chromatogram obtained in the present study. The profile corresponds to rat plasma containing about 1 μg ml^{-1} of the drug. Similar chromatograms were obtained for human plasma. The peak of heptaminol-NBD appears with an R_F of 0.45 in the valley between two smaller peaks that correspond to those of impurities of the reagent NBD-Cl. The large peak that appears at the R_F value of 0.32 also corresponds to that of an impurity of the reagent, which does not interfere with the determination of heptaminol-NBD.

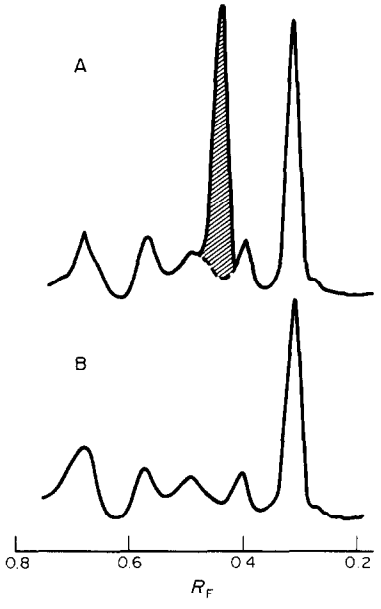

Figure 1
Characteristic thin-layer fluorescence chromatograms ($\lambda_{excitation}$ = 450 nm; $\lambda_{emission}$ = 525 nm) of: (A) rat plasma extract containing the heptaminol-NBD derivative (corresponding to a concentration of heptaminol in plasma of about 1 μg ml^{-1}); and (B) rat blank plasma. Both chromatograms were developed in chloroform–methanol (90:10, v/v). The volume of plasma analysed was 0.5 ml.

The plot of derivative peak area against concentration of heptaminol in plasma is a straight line for a concentration range of 50–5000 ng ml^{-1} (using 0.5 ml of plasma): $y = 610.5\,x$, where y is the peak area (mm^2) and x is the concentration (μg ml^{-1}) in plasma (SE of the gradient = 29.8; n = 6 curves on different days, with correlation coefficients greater than 0.996). The sensitivity limit of the method under the conditions described in the experimental section was estimated to be about 25 ng ml^{-1} in human plasma when 1.0 ml is analysed.

The precision of the method was determined by assaying in sextuplicate human plasma samples containing 200 ng ml^{-1} of heptaminol. The relative standard deviation was 6.1%.

The yield of the extraction process from plasma was calculated by comparing the fluorescence intensity mean values obtained from six extracted samples with that obtained from six standard solutions directly treated with NBD-Cl. A mean yield of 80.9 ± 2.0% (± SE) was obtained at the concentration studied (200 ng ml^{-1}).

The usefulness of this method for pharmacokinetic studies is reflected in Fig. 2 which shows the plasma concentrations in rats after oral administration of doses of 5 mg kg^{-1} (about twice the human therapeutic dose). The mean half-life of heptaminol elimination was approximately 63 min.

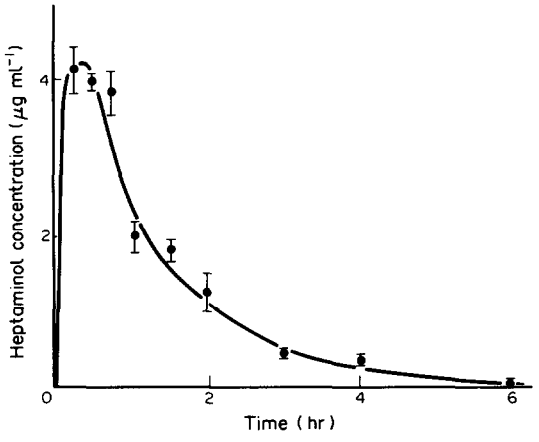

Figure 2
Concentrations of heptaminol expressed as μg ml^{-1} of heptaminol free base in plasma of rats killed at different times after administration of 5.0 mg. kg^{-1} of the drug by stomach tube. Each point represents the mean concentration in three rats and the vertical bars correspond to the standard error of the mean.

The precise chemical structure of the fluorescent derivative, elucidated by spectroscopic methods, is 6-(7′-nitrobenzo-2′,1′,3′-oxadiazo-4-yl)-amino-2-methyl-2-heptanol. Such a structure is in accordance with the mass of the molecular ion (M^+ at *m/e* 308) and of the ions produced (see mass spectrum in Fig. 3a) by fragmentations at the more substituted points of the hydrocarbon framework (*m/e* 207 and *m/e* 59). The absence of isotopic doublets of chlorine in the mass spectrum indicates that carbon 4 is the substituted position in NBD−. This fact is confirmed by the upfield shift of the doublet of the proton in position 5′ (δ 6.23 ppm) in the nuclear magnetic resonance spectrum of the derivative (Fig. 3b) with regard to the position occupied in the NBD-Cl spectrum (δ 7.65 ppm). Furthermore, infrared spectra (Fig. 3c), fluorescence spectra (excitation max. 475 nm, emission max. 510 nm; see Fig. 3d) and results of elemental analysis (calculated %: C 54.55, H 6.49, N 18.18; experimental %: C 53.49, H 6.29, N 17.65) are also in accordance with the proposed structure.

Discussion

On the basis of the reaction of primary amines with NBD-Cl, a method of plasma extraction and measurement of fluorescence on thin-layer plates was developed by Van Hoof and Heyndricks [7] for the analysis of a series of sympathomimetic drugs which included heptaminol. In the present work, the recovery of heptaminol was less than 30%.

The main cause for this low recovery was the pH used for the extraction of the drug (pH 9.0). Because the pK_a of heptaminol is 10.6, this compound is mainly in the ionized form at pH 9.0; thus effective extraction by organic solvents is prevented. For this reason, in place of borate buffer (pH 9.0), 0.1 ml of 2N NaOH was added to the samples to give a final plasma pH of 12.0–12.5. Nevertheless since unionized heptaminol is a volatile compound, evaporation of the ethereal extract must be carried out at a pH in which the compound is completely ionized (below 8). The use of an aqueous solution of HCl to reduce the pH of the etheral phase is not practical because of the subsequent

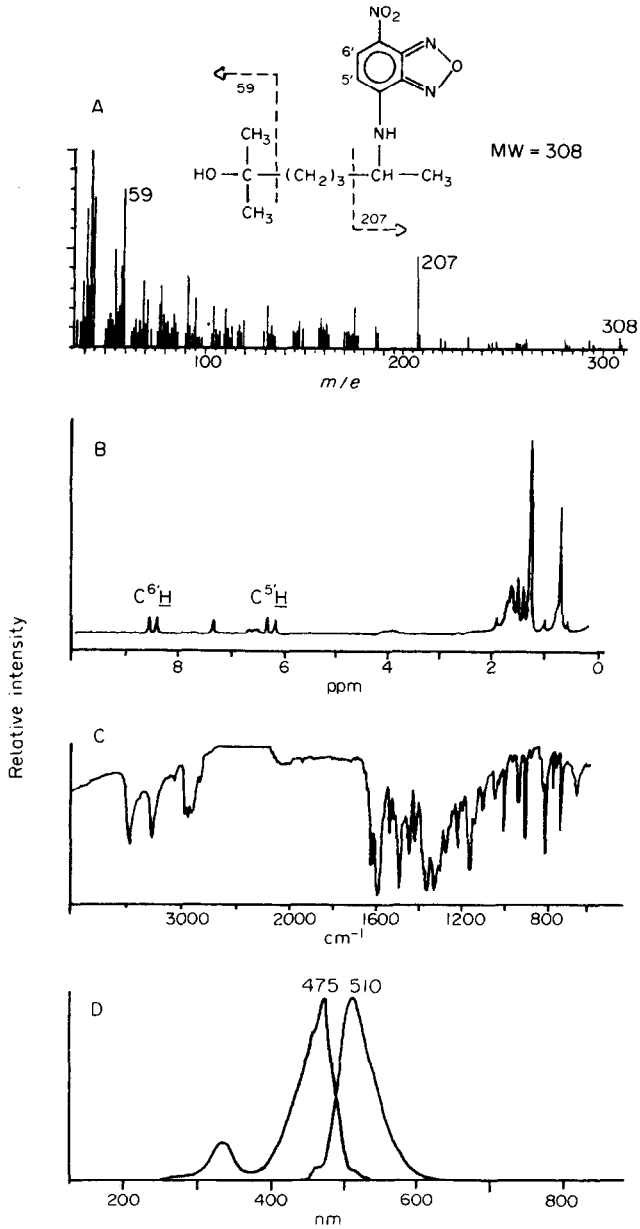

Figure 3
Structure and (A) mass, (B) nuclear magnetic resonance, (C) infrared, and (D) fluorescence spectra of heptaminol-NBD derivative.

difficulty of eliminating water and excess HCl. Therefore this step was replaced by the use of ether saturated with HCl; in this way a dry residue free of HCl was obtained, as required for the reaction of heptaminol with NBD-Cl.

MIBK is a suitable solvent for the coupling reaction because of its high polarity and boiling point [8]. However, the use of this solvent for the application of samples to the thin-layer plates yielded large spots with a resultant loss of sensitivity. Replacement of this solvent by chloroform gave much smaller spots and consequently provided sharper peaks. The solvent systems described previously [7] for the thin-layer chromatography of NBD derivatives of sympathomimetic drugs were unsuitable for the chromatography of the heptaminol derivative; all solvent systems produced low R_F values and the derivative spot was superimposed with other interfering products. On the basis of empirically established eluotropic series [9] six solvent pairs were tested (Table 1). System number 2 was chosen to give minimal superimposition of the heptaminol derivative spot with impurities, by-products or excess reagent.

Table 1
R_F values of the heptaminol–NBD derivative obtained by thin-layer chromatography in different solvent systems.

Code No.	Solvent system (v/v)	R_F	Comments
1	Ethyl acetate–cyclohexane (7:3)	0.23	Too low R_F. Interference
2	Chloroform–methanol (9:1)	0.45	Very good separation
3	Benzene–ethanol (3:1)	0.50	Good separation
4	Chloroform–acetone (1:1)	0.49	Good separation
5	Chloroform–methanol (4:1)	0.54	Slight overlapping with excess reagent
6	Methanol–ethyl acetate (7:3)	0.73	Total overlapping with excess reagent

As described in the experimental procedure plates were stored in the dark before being analysed. Nevertheless, it has been verified that light does not significantly influence the measurements. Indeed, spot fluorescence emission is not modified after exposure to light of 450 nm wavelength from a mercury lamp for 80 min. This means that consecutive scannings of a spot will not give different results as occurs with other NBD derivatives [8].

The fluorescence spectrum of the NBD-heptaminol derivative (Fig. 3d) shows a maximum of excitation at 475 nm for a maximum emission at 510 nm. The photodensitometer was used with filters of 450 nm for excitating light and 525 nm for emitted light and it has been verified that, under these conditions, the sensitivity of detection is 55% of that obtained using the respective maximum wavelengths. Thus, the sensitivity of the method for an instrument provided with a variable monochromator should be about twice that found in the present study.

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