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Short communication

High-performance liquid chromatographic procedure for the measurement of nitrobenzodiazepines and their 7-amino metabolites in blood

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Abstract

A simple and sensitive HPLC method is described for the determination of the nitrobenzodiazepines, nitrazepam, flunitrazepam and clonazepam and their respective 7-amino metabolites in post-mortem blood. Using a single-step extraction the nitrobenzodiazepines were recovered from 0.5 ml of blood using butyl chloride. The solvent was evaporated to dryness and the reconstituted residue injected onto an HPLC system. Separation was achieved using a phenyl-bonded reversed-phase column with an acetonitrile–phosphate buffer mobile phase. Ultraviolet detection (240 nm) was used for quantitation. A linear response was observed between 0.01 and 0.60 mg/l. Assay precision between and within assays was less than 20% for all analytes. The limits of detection ranged from 0.001 to 0.008 mg/l with a limit of quantitation of 0.01 mg/l for all analytes.

1. Introduction

The benzodiazepine family of drugs is now the leading group of drugs prescribed for the management of anxiety throughout the world. Flunitrazepam, clonazepam and nitrazepam are all nitro-containing benzodiazepines prescribed widely in Australia for either sleep disorders, anxiety or epilepsy.

All these drugs are at least in part metabolised during life to the 7-amino metabolites, 7-aminonitrazepam (7AN), 7-aminoclonazepam (7AC) and 7-aminoflunitrazepam (7AF) [1–3]. Post-mortem bioconversion to 7-amino metabolites may also occur, to the extent that little or no parent drug may be present [3–5]. The identification and quantitation of the 7-amino metabolites are of importance toxicologically as they are often the only indication of nitrobenzodiazepine use prior to death [4,5]. Consequently the determination and quantitation of these drugs and their respective metabolites is of clinical and forensic interest.

A number of analytical methods for the determination of benzodiazepines in biological fluids have been published previously [6–12]. Only one method has been described for the detection of all nitrobenzodiazepines and metabolites [12]. This method which was developed in our laboratories involves the use of a gradient

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programming method and diethyl ether as extraction solvent. Since the use of ether provides a significant occupational health and safety risk to laboratory workers alternative solvents are preferred.

We have developed a comparatively simple HPLC–UV method for the rapid and sensitive determination of nitrobenzodiazepines and their respective 7-amino metabolites in blood without the use of ether. This method can be used to provide a second method for the confirmation and quantitation of these and other benzodiazepines after their preliminary identification on other chromatographic systems [13].

2. Experimental

2.1. Reagents and glassware

All drugs and metabolites were obtained from the curator of standards at the Australian Government Analytical Laboratories.

Sodium carbonate (Na_2CO_3) and potassium dihydrogen orthophosphate (KH_2PO_4) were of analytical reagent grade (Ajax Chemicals, Australia). Acetonitrile (Mallinckrodt, Australia), butyl chloride (Fisons, UK) were of HPLC grade. Extraction tubes were silanised by immersing the glassware in a 5% solution of Surfasil (Pierce Chemical Company, USA) in toluene for 1 h, followed by rinsing in methanol. These were then dried before use.

2.2. Standards and controls

Stock drug solutions were prepared in methanol at a concentration of 1 mg/ml. Working standard solutions were prepared by adding dilutions of the stock solutions with methanol to out-of-date blank blood to give final concentrations ranging from 0.01 to 0.60 mg/l.

Blood specimens prepared with known concentrations of nitrobenzodiazepines (controls) were assayed in each experiment to provide a measure of quality assurance.

2.3. Chromatography

A mobile phase of acetonitrile–40 m*M* phosphate (KH_2PO_4) buffer (28:72, v/v), pH 3.75, was used after filtration and degassing (0.45-μm pore filter, Waters Millipore, Australia). Phosphate buffer was prepared by dissolving 5.44 g KH_2PO_4 in 1 l of deionised water. The flow-rate was 0.8 ml/min using a Shimadzu LC-6AD pump. Separation was achieved using a 4-μm phenyl-bonded (60 Å, 150 × 3.9 mm I.D.) Waters Nova-Pak column. Sample injection was by a Shimadzu SIL-6B auto-injector, and detection was achieved by a SPD-6AV UV-Vis variable-wavelength spectrophotometric detector operated at 240 nm (Shimadzu Oceania, Australia). Chromatograms were generated using a CR4-A integrator/plotter (Shimadzu Oceania, Australia) with peak height and retention time recordings. All instrumentation were operated at ambient temperature.

2.4. Extraction procedure

To 500 μl of blood in silanised 10-ml glass extraction tubes, 15 μl of the internal standard demoxepam (10 μg/ml) and 500 μl of 0.2 *M* carbonate buffer (pH 11.5) were added successively with mixing followed by 6 ml of butyl chloride. The tubes were rotated gently for 20 min and then centrifuged at 2500 *g* for 10 min. Following extraction the organic layer was transferred to a clean 10-ml glass extraction tube and evaporated to dryness in a sample concentrator (Savant Industries, Selby's Australia Pty). The residue was reconstituted with 200 μl of mobile phase and the extract was transferred to autosampler vials containing micro glass inserts. A 50-μl aliquot was injected onto the HPLC system.

2.5. Validation

Peak-height ratios of the six analytes to the internal standard were calculated to generate standard calibration curves of peak-height ratio versus drug concentration. Linear regression analysis was performed to determine the intercept, slope and correlation coefficient of the calibration lines.

Coefficients of variation (C.V.) for intra-assay precision were calculated by spiking known amounts of drug into blood. Six samples were determined at three different concentrations for each drug (Table 3). The inter-assay precision was evaluated by analysing duplicate samples, at the same concentrations, on six different days.

Recoveries of the various analytes were calculated by comparing the peak-height response of a known extracted standard to the peak heights of known amounts of unextracted standards prepared in mobile phase.

3. Results and discussion

Fig. 1 shows typical chromatograms of an extracted blank blood specimen, a blood standard (containing the additional internal standard temazepam) and a post-mortem blood specimen. These chromatograms show good chromatographic resolution and separation of each of the nitrobenzodiazepines and their metabolites. Extracts of fresh blood and post-mortem blood were both found to be free of significant interference from endogenous peaks. Post-mortem blood specimens taken from decomposing bodies were also not found to contain interfering peaks.

Elution of the nitrobenzodiazepines occurred over a run time of 25 min. Retention times are shown in Table 1. The limits of detection, defined as 3 times the background noise, were found to be between 0.001 and 0.008 mg/l for all analytes measured (Table 1). The limit of quantitation for all analytes was 0.01 mg/l.

The recoveries of the analytes ranged from 53% for 7AN to 100% for the internal standard demoxepam (Table 1). These recoveries were twice that obtained for the diethyl ether method using a back-extraction clean-up [12].

A further eight commonly encountered benzodiazepines and 13 other drugs known to elute in a gradient benzodiazepine assay [12] did not interfere with the analysis of the nitroben-

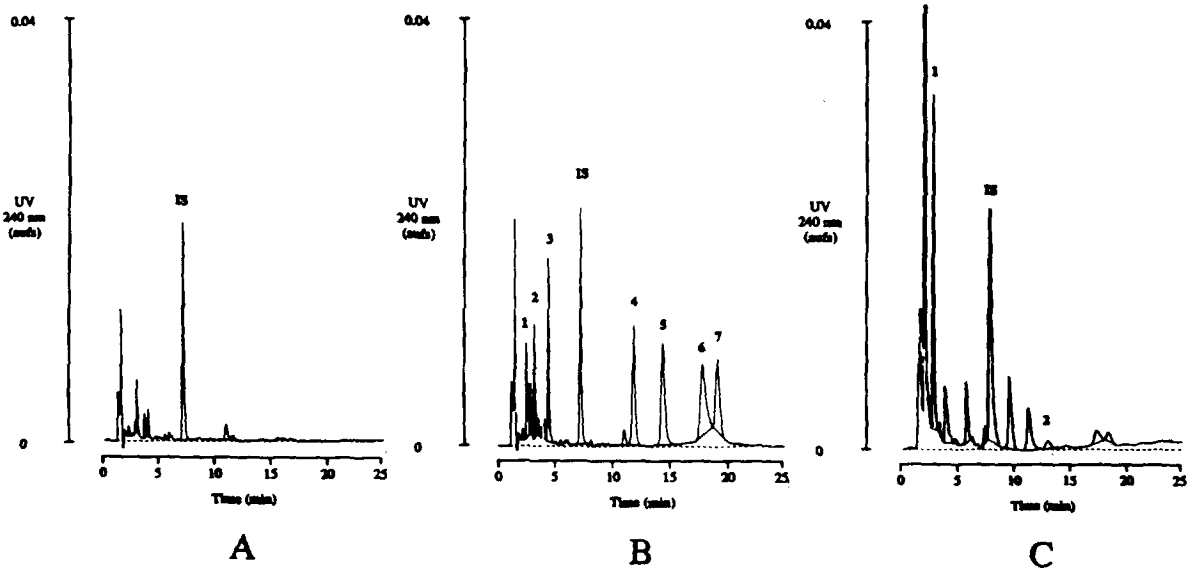

Fig. 1. Chromatograms showing UV responses to (A) an extracted blank blood specimen with 0.15 mg/l internal standard (I.S.) (demoxepam), (B) an extracted blood standard with metabolites 7-aminonitrazepam (1), 7-aminoclonazepam (2), 7-aminoflunitrazepam (3) at 0.10 mg/l, and the parent drugs nitrazepam (4), clonazepam (5), temazepam (6) and flunitrazepam (7) at 0.20 mg/l and I.S. at 0.15 mg/l, (C) an extracted post-mortem blood specimen showing 7-aminonitrazepam (1, 0.23 mg/l) and nitrazepam (2, 0.01 mg/l) and I.S. at 0.15 mg/l.

Table 1
Relative retention times, recovery data and detection limits of nitrobenzodiazepines and metabolites

Compound	RRT[a]	Recovery[b] (%)	Detection limit (mg/l)
7-Aminonitrazepam	0.305	53	0.003
7-Aminoclonazepam	0.420	67	0.003
7-Aminoflunitrazepam	0.560	98	0.001
Demoxepam (I.S.)	1.000	100	0.001
Nitrazepam	1.605	100	0.008
Clonazepam	1.995	100	0.006
Flunitrazepam	2.640	100	0.008

[a] Demoxepam = 1.000, eluting at 8.00 ± 0.05 min.
[b] Mean of 8 experiments.

zodiazepines and their metabolites (Table 2). This assay could therefore also be used for the quantitation of these other benzodiazepines. Relative retention times were reproducible and varied on average by less than 0.5% from run to run.

Coefficients of variation (C.V.) for intra-assay and inter-assay variability are shown in Table 3. The variations for intra-assay were less than 20% for the low standard (0.01 mg/l) and less than 10% for the medium and high standards (0.10 and 0.40 mg/l). The variations for the inter-assay were less than 10% for all analytes at the 3 concentrations (0.05, 0.25 and 0.50 mg/l).

Calibration curves over the range 0.01–0.60 mg/l were found to be linear for all analytes. Typical linear regression equations for the analytes 7AN, 7AC and 7AF were: $y = 15 + 0.03x$ ($r^2 = 0.999$), $y = 0.6 + 0.02x$ ($r^2 = 1.000$) and $y = 9 + 0.01x$ ($r^2 = 1.000$), respectively, with nitrazepam, clonazepam and flunitrazepam, $y = 7 + 0.04x$ ($r^2 = 1.000$), $y = 14 + 0.04x$ ($r^2 = 0.999$) and $y = 15 + 0.07x$ ($r^2 = 0.998$), respectively.

This method has been successfully used for the quantitation of 7-amino metabolites, produced in post-mortem blood by bacteria, and for the detection of nitrobenzodiazepines and their metabolites in post-mortem blood from cadavers. In 34 benzodiazepine-positive cases nitrazepam was detected in 5 cases (range 0.01–0.26 mg/l) and 7AN in 12 cases (range 0.02–0.98 mg/l). Clonazepam was detected in 5 cases (0.03–0.23 mg/l) and 7AC in 11 cases (0.02–1.83 mg/l). Flunitrazepam was not detected in any case, however 7AF was detected in 14 cases (0.01–0.23 mg/l). In no case was a nitrobenzodiazepine detected without its respective 7-amino metabolite and in all cases the concentration of the 7-amino metabolite was greater than for the respective parent nitrobenzodiazepine. Only 28% of the cases contained the parent nitrobenzodiazepine. These data therefore reinforce the need to measure the 7-amino metabolite as

Table 2
Relative retention times of other benzodiazepines and drugs known to elute under similar conditions

Benzodiazepine		Other drugs	
Compound	RRT[a]	Compound	RRT[a]
Bromazepam	0.741	Theophylline	<0.250
Oxazepam	1.422	Paracetamol	<0.250
Lorazepam	1.602	Salicylic acid	0.335
Alprazolam	1.966	Moclobemide	0.457
Triazolam	2.024	Carbamazepine epoxide	0.596
Desalkylflurazepam	2.058	Phenobarbitone	0.611
Desmethyldiazepam	2.178	Quinine	0.712
Temazepam	2.460	Quinidine	0.766
Flurazepam	2.666	Carbamazepine	0.943
Diazepam	3.901	Phenytoin	1.291
		Naproxen	2.869

[a] Demoxepam = 1.000, eluting at 8.00 ± 0.05 min.

Table 3
Accuracy and precision data for nitrobenzodiazepines and metabolites

Compound	Concentration added (mg/l)	Concentration detected[a] (mg/l)	C.V. (%)
Intra-assay (n = 6)			
7-Aminonitrazepam	0.01	0.010 ± 0.001	10
	0.10	0.097 ± 0.003	3.1
	0.40	0.37 ± 0.02	5.4
7-Aminoclonazepam	0.01	0.008 ± 0.001	13
	0.10	0.093 ± 0.004	4.3
	0.40	0.37 ± 0.02	6.5
7-Aminoflunitrazepam	0.01	0.010 ± 0.001	10
	0.10	0.095 ± 0.005	5.3
	0.40	0.36 ± 0.02	4.4
Nitrazepam	0.01	0.009 ± 0.001	11
	0.10	0.096 ± 0.004	4.2
	0.40	0.32 ± 0.03	8.4
Clonazepam	0.01	0.013 ± 0.001	7.7
	0.10	0.101 ± 0.004	4.0
	0.40	0.35 ± 0.03	8.6
Flunitrazepam	0.01	0.011 ± 0.001	9.1
	0.10	0.107 ± 0.004	3.7
	0.40	0.40 ± 0.01	3.3
Inter-assay (n = 6)			
7-Aminonitrazepam	0.05	0.050 ± 0.004	8.0
	0.25	0.26 ± 0.02	6.1
	0.50	0.51 ± 0.02	4.5
7-Aminoclonazepam	0.05	0.052 ± 0.002	3.8
	0.25	0.24 ± 0.02	10
	0.50	0.50 ± 0.03	5.1
7-Aminoflunitrazepam	0.05	0.051 ± 0.001	2.0
	0.25	0.24 ± 0.01	6.2
	0.50	0.47 ± 0.02	5.1
Nitrazepam	0.05	0.050 ± 0.003	6.0
	0.25	0.24 ± 0.01	2.8
	0.50	0.51 ± 0.02	4.5
Clonazepam	0.05	0.051 ± 0.003	5.9
	0.25	0.24 ± 0.01	5.7
	0.50	0.51 ± 0.03	5.8
Flunitrazepam	0.05	0.052 ± 0.004	7.7
	0.25	0.25 ± 0.02	6.6
	0.50	0.50 ± 0.03	5.3

[a] Mean ± S.D.

well as the parent nitrobenzodiazepine in post-mortem specimens [5].

This procedure is also being used by this laboratory as a modification of the method by McIntyre et al. [12] for the successful identification and quantitation of all 15 benzodiazepines. This involves substituting the isocratic conditions described here for the gradient conditions described in McIntyre et al. [12].

In conclusion, a rapid, sensitive and reproducible HPLC technique for the detection and quantitation of the nitrobenzodiazepines and their respective metabolites is described. This method is useful for both ante-mortem and post-mortem blood. This method obviates the need for the flammable diethyl ether to be used as extraction solvent and allows for the quantitation of a number of other commonly encountered benzodiazepines.

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