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Note

Analysis of nitrazepam and its metabolites by high-pressure liquid chromatography

B. MOORE and G. NICKLESS
Department of Inorganic Chemistry, University of Bristol, Bristol, Avon (Great Britain)
C. HALLETT
Roche Products Ltd., Broadwater Road, Welwyn Garden City, Hertfordshire (Great Britain)
and
A. G. HOWARD*
Department of Chemistry, The University, Southampton SO9 5NH, Hants (Great Britain)
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The benzodiazepines are a widely used class of drugs possessing sedative and muscle-relaxant properties. The most generally applicable procedure for the analysis of benzodiazepines in biological fluids, such as blood and urine, involves a multi-stage solvent extraction, followed by gas–liquid chromatographic assay[1–3].

Gas chromatography of diazepam, medazepam, oxazepam and their metabolites can be satisfactorily performed on silicone phases such as OV-1, OV-17 and OV-225[3,4]. Nitrazepam and its metabolites, however, fail to chromatograph satisfactorily, giving rise to severely tailing peaks. It is therefore necessary to hydrolyse these compounds to their corresponding benzophenones[5] or to convert nitrazepam to its methyl derivative[6] prior to chromatography. These methods suffer from the drawback that neither of the major metabolites of nitrazepam, 7-aminonitrazepam and 7-acetamidonitrazepam can be separately determined.

Analyses of benzodiazepine mixtures by high-pressure liquid chromatography (HPLC) have been reported by several authors. Mixtures of intact benzodiazepines have been separated on silica[7], cation-exchange resins[8], Durapak OPN[9] and Carbowax 400 coated support[10].

Although many procedures have been developed for the separation of nitrazepam from other benzodiazepines, no technique has been reported for the chromatographic resolution of nitrazepam and its metabolites by gas or liquid chromatography. This note describes such an analysis performed by HPLC on an anion-exchange packing.

EXPERIMENTAL

The liquid chromatograph consisted of twin Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 reciprocating piston pumps, controlled by a Waters Model

* To whom correspondence should be addressed.

660 solvent programmer. Column effluent was monitored by a Varian (Walnut Creek, Calif., U.S.A.) Model 635 M spectrophotometer, equipped with twin 8-μl flow cells. The detector was operated at 260 nm with a slit width of 2 mm.

The column consisted of a 50 cm × 2 mm I.D. stainless-steel tube, dry packed with the strong anion-exchange material, Zipax SAX (30 μm mean particle diameter; DuPont, Wilmington, Del., U.S.A.). Sample injection was made directly onto the column via a septum injection port.

Reagents

Reagent-grade ethyl acetate and hexane were obtained from BDH (Poole, Great Britain). These were redistilled and degassed by boiling prior to use. Benzodiazepines were obtained from Roche Products (Welwyn Garden City, Great Britain).

METHOD AND RESULTS

Extraction

An amount of 10 ml of human urine, adjusted to pH 7 with 1 *M* acetate buffer,

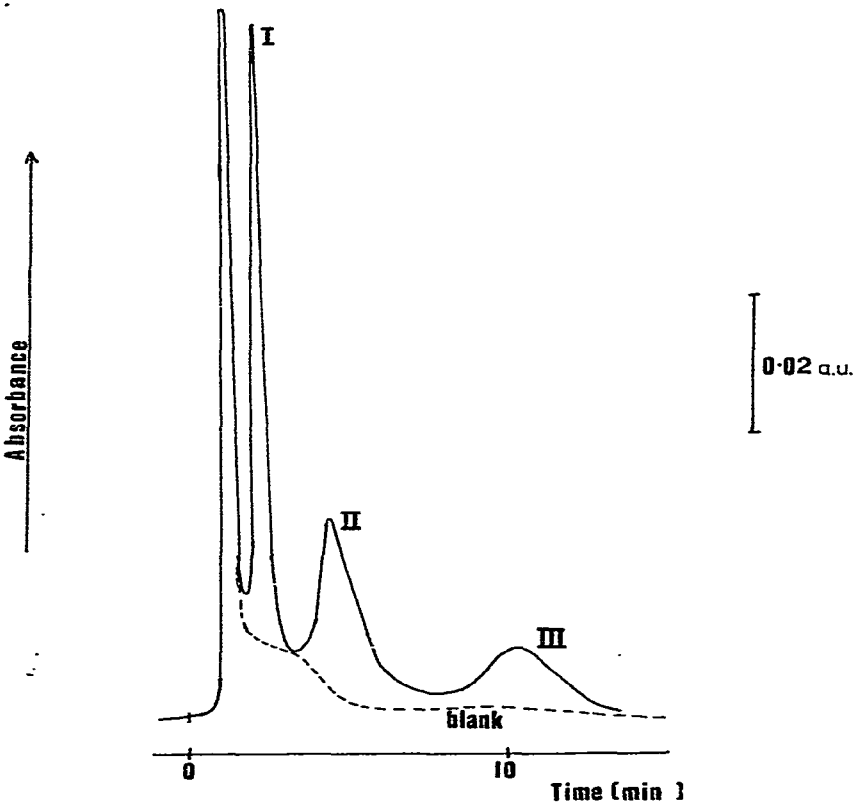

Fig. 1. HPLC separation of nitrazepam and metabolites on Zipax SAX. – – –, Urine blank (5 μl of extract injected onto column); ———, extract from urine spiked with 10 μg/ml nitrazepam and metabolites (5 μl injected onto column). Peaks: I = nitrazepam; II = 7-aminonitrazepam; III = 7-acetamidonitrazepam.

was extracted three times with 10-ml portions of ethyl acetate. The combined extracts were evaporated to dryness and redissolved in 1 ml of ethyl acetate. Recovery of spiked nitrazepam and metabolites from urine was 80 ± 4% at concentrations of 10 μg/ml.

Chromatography

Benzodiazepine samples were injected onto a column of Zipax SAX and eluted with a solvent mixture consisting of ethyl acetate–hexane (3:7) at a flow-rate of 1 ml/min. A typical chromatogram of an extract obtained from urine spiked to a level of 10 μg/ml with nitrazepam and its metabolites is given in Fig. 1. Detector response was found to be linear for all three compounds over the range 0–700 ng. Detection limits were in the range 20–100 ng (peak height = 2 × noise signal), corresponding to 0.4–2.0 μg of benzodiazepine per millilitre of urine.

DISCUSSION AND CONCLUSIONS

Nitrazepam and its metabolites have been analysed by HPLC on a column of strong anion-exchange resin. The mechanism for this separation is poorly understood but has previously been observed with molecules containing substituted nitrogen moieties[11].

The technique is rapid, moderately sensitive and, unlike gas chromatographic analysis, allows separation and quantification of both nitrazepam and its metabolites without the necessity for an hydrolysis stage. It has been demonstrated that the procedure is applicable to the analysis of relatively high levels of nitrazepam in urine, but a more efficient multi-stage clean-up procedure, such as is used for gas chromatographic analysis, may be required for more dilute samples.

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