Journal of Chromatography, 276 (1983) 218—223
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1708

Note

Paired-ion extraction and high-performance liquid chromatographic determination of diminazene in plasma

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(Received January 21st, 1983)

Diminazene (4,4'-diamidinodiazoaminobenzene diacetamidoacetate) (Berenil; Hoechst, Frankfurt a.M., G.F.R.) is used extensively in the treatment of trypanosomiasis and babesiosis in animals [1—5]. It has also been used in clinical trials in early cases of human sleeping sickness [6].

Several techniques have been reported for the quantitative extraction and determination of diminazene in plasma. These methods include biological assay [7], paper and thin-layer chromatography [8], colorimetry [9], high-performance liquid chromatography (HPLC) [10], gas chromatography—chemical ionization mass spectrometry (MS) [11] and radiometry using the ^{14}C-labelled drug [12]. The bioassay, colorimetric and radiometric methods lack sufficient specificity as they determine the total of all biologically active or radioactive components, and normal plasma may give a significant colour reaction when diazotized. The paper and thin-layer chromatographic methods are not sufficiently sensitive to detect submicrogram concentrations, while the HPLC and MS methods, although sensitive, involve tedious and protracted sample preparation steps, including the reduction of diminazene to 2 mole of 4-aminobenzamidine prior to extraction, derivatization and analysis [10, 11].

In animals treated with diminazene, there may be metabolites which are structurally unknown or closely related to the parent drug or the reduction products. An analytical method suitable for pharmacokinetic studies of diminazene must include an efficient extraction of the parent compound from plasma and other body fluids. The ion-pair extraction method and assay by reversed-phase HPLC described in this paper is specific for the intact diminazene molecule and is suitable for its pharmacokinetic evaluation.

0378-4347/83/$03.00

EXPERIMENTAL

HPLC instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) modular HPLC system was used; it consisted of a constant-flow solvent delivery pump (Model M-6000A) and a WISP injector (Model 710B); separation of injected compounds was achieved by a Radial-PAK CN column (10 cm × 5 mm I.D., 10 μm particle size) fitted with a CN guard column. The column effluent was monitored with a 254-nm detector (Model 440) operated at 2×10^{-2} absorbance units full scale (a.u.f.s.); areas and concentrations of peaks were determined by an on-line computer (Waters Data Module M-730). Mobile phase was pumped at a rate of 0.8 ml min^{-1}. The cartridge (Sep-Pak C_{18}) which was used to extract diminazene from plasma was supplied by Waters Assoc.

Reagents

Berenil and diminazene diaceturate were supplied by Hoechst and imidocarb dipropionate by Burroughs Wellcome (London, Great Britain). Methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Great Britain) and were of HPLC grade. Triethylamine was supplied by Fluka (Buchs, Switzerland). Orthophosphoric acid was supplied by Koch-Light (Colnbrook, Great Britain). 1-Heptanesulphonic acid was supplied as Pic B-7 reagent by Waters Assoc.

HPLC mobile phase

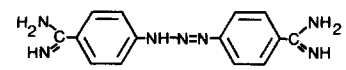
The mobile phase used for isocratic elution of diminazene and the internal standard consisted of acetonitrile and glass-distilled deionized water (50:50, v/v) to which was added 0.2% of triethylamine. The pH was adjusted to 4.20 with orthophosphoric acid.

Standard solutions

Stock solutions of diminazene diaceturate and the internal standard, imidocarb dipropionate (100 μg ml^{-1} each), were prepared in glass-distilled water. A series of standard solutions (2.5—250 ng of diminazene containing 15 ng of the internal standard per 50 μl injected) were then prepared in methanol–heptanesulphonic acid for use in establishing the linearity of the assay procedure.

Extraction procedure

General concept. The method utilized a cartridge with C_{18} reversed-phase packing material. After applying the sample to the cartridge it was washed under conditions not eluting diminazene and the internal standard. An ion pair was then formed with a suitable counter ion and eluted from the cartridge with a solvent with moderate solvating ability. The HPLC analysis was carried out according to the concept of internal standardization in the plasma sample. The internal standard, imidocarb [3,3′-di(2-imidazolin-2-yl)carbanilide] was selected as it is closely related chemically to diminazene (Fig. 1) and was readily extracted and quantitated by the same method.



I

II

Fig. 1. Diminazene (I) and the internal standard, imidocarb (II).

Sample treatment. To 1.0 ml of plasma were added internal standard solution equivalent to 6 μg of imidocarb base and an aliquot of the stock diminazene solution equivalent to 0.05—5.0 μg ml^{-1} of plasma. The sample was mixed on a Vortex mixer and then half the volume was passed through the Sep-Pak C_{18} cartridge fitted to a Luer Lock syringe. The cartridge was pre-washed with 2 ml of methanol and 5 ml of distilled water. After washing with 2 ml of each of 20% methanol in distilled water and methanol, 1 ml of 0.025 *M* 1-heptanesulphonic acid in 90% methanol in distilled water was used to elute diminazene and imidocarb. The solvent was allowed to drip through the cartridge from the syringe without applying pressure, except the last few drops, which were forced through by air applied from the syringe. The effluent was vortexed and 50 μl were injected into the HPLC system.

Quantitative evaluation and sensitivity

The recovery of diminazene from plasma was determined by comparing the amounts resulting from spiked plasma samples to those obtained from direct injection of similar concentrations of diminazene in aqueous solution.

RESULTS

Chromatograms

Sample chromatograms of blank plasma, of plasma collected from a goat 1 h after an intravenous dose of diminazene (2.0 mg kg^{-1}), of plasma fortified with both diminazene and imidocarb and of diminazene are presented in Fig. 2. Diminazene and imidocarb are well separated with retention times (t_R) of 5.08 and 6.60 min, respectively, and a separation factor (α) of 1.475. Neither the solvent front nor coextracted endogenous plasma compounds interfered with the measurement of either compound. About 2.0 μg ml^{-1} of diminazene and 1.0 μg ml^{-1} of the internal standard gave about 50% full-scale response at 2 × 10^{-2} a.u.f.s. The peaks were well resolved with peak resolutions (R_s) of 5.32 and 4.38 for diminazene and imidocarb, respectively.

Statistical validation of the method

The results of the intra-assay linearity, precision and sensitivity limit of the method are summarized in Table I. The response of diminazene was linear over the range of concentrations studied (0.05—5.0 μg ml^{-1}) ($r^2 > 0.998$). The recovery from plasma was 92.0 ± 7.8% (S.D.) with a mean coefficient of variation of 6.5%. The limit of quantification was 50 ng ml^{-1} in plasma, without any concentration step.

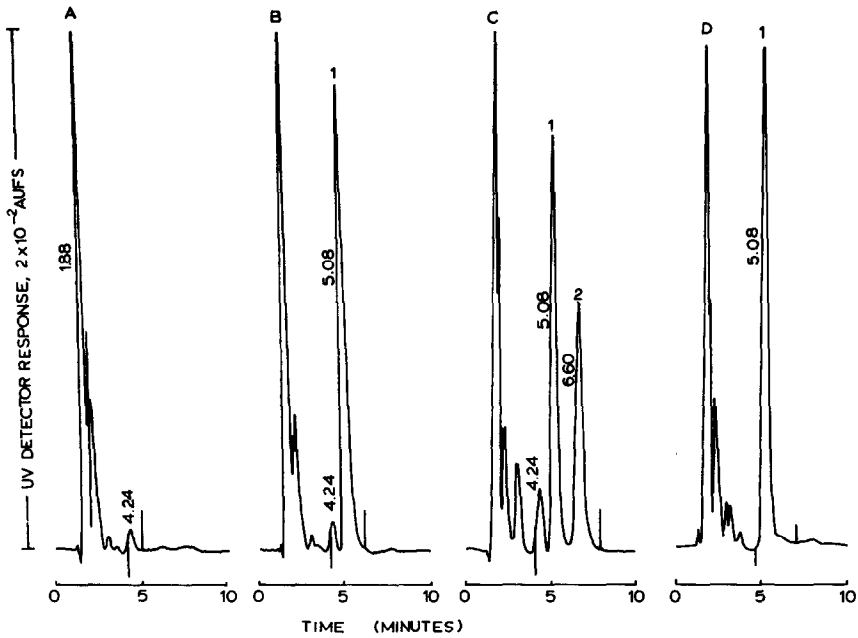


Fig. 2. HPLC traces of (A) blank plasma; (B) goat plasma 1 h after a 2.0 mg/kg intravenous dose of diminazene; the peak corresponds to a concentration of 3.4 μg/ml; (C) plasma fortified with authentic diminazene (3.0 μg/ml) and imidocarb (1 μg/ml); (D) aqueous solution of authentic diminazene (3.8 μg/ml) injected directly into the HPLC system. Peaks: 1, diminazene; 2, internal standard (imidocarb).

TABLE I

LINEARITY OF THE ASSAY PROCEDURE AND RECOVERY OF DIMINAZENE FROM PLASMA

Spiked concentration (μg)	Number of assays	Recovery (mean ± S.D.) (μg)	Recovery (%)
0.05	3	0.039 ± 0.005	77.3
0.10	5	0.079 ± 0.010	79.4
0.20	3	0.18 ± 0.021	91.7
0.30	3	0.27 ± 0.031	88.9
0.40	3	0.36 ± 0.020	90.4
0.50	5	0.47 ± 0.046	93.0
1.0	7	0.97 ± 0.077	96.0
2.0	3	1.94 ± 0.065	96.8
3.0	7	2.92 ± 0.105	97.2
4.0	3	3.94 ± 0.021	98.6
5.0	5	5.13 ± 0.153	102.7
Mean ± S.D.			92.0 ± 7.8

Application of the method

The method is sufficiently accurate to permit the determination of approximately 1/150th the peak plasma level following single therapeutic doses of Berenil in goats. The method has also been used to follow milk and urine levels

of diminazene and its partition between plasma and erythrocytes following single intravenous injections in goats. Commercially available Berenil was dissolved in the sulphonic acid—methanol solution to a nominal concentration, i.e., the manufacturer's value of 3.0 μg ml^{-1}, and injected into the HPLC system for the quantitation of its diminazene content. The amount of diminazene in the formulation, as established by the present method, was 2.97 ± 0.05 μg ml^{-1}. Diminazene (t_R = 5.08 min) was well separated from the phenazone (t_R = 2.84 min) content of Berenil.

DISCUSSION

Sensitive HPLC and MS systems have been utilized by Fouda [10, 11] for determining diminazene, but the intact drug could not be extracted from plasma and had to be split into 2 mole of 4-aminobenzamidine, which were extracted, derivatized and analysed. In the present method, the extraction of intact diminazene from plasma using a cartridge packed with reversed-phase material was found to be simple and quantitative. The plasma extract was sufficiently clean to be injected directly into the HPLC system. Each cartridge was used 7—9 times without loss of efficiency.

Diminazene exhibited a high affinity for the stationary phase of the cartridge as it could not be eluted by water or organic solvents such as methanol, acetonitrile, diethyl ether, or dichloromethane. However, it was readily eluted by a small volume of methanol to which a counter ion was added. This indicated that diminazene is present in aqueous solutions mainly in the ionized form, as can also be predicted by the presence of both primary amine and imino groups in its structure. The traditional approach of solvent extraction in the uncharged form may therefore not succeed, making ion-pair extraction a good alternative [13]. Three counter ions were found to be suitable: (1) a mixture of perchloric acid (0.2 *M*) and sodium perchlorate (0.8 *M*); (2) tetrabutylammonium hydrogen sulphate (0.02 *M*); and (3) 1-heptanesulphonic acid (0.025 *M*) in 90% methanol in water. Eluates of the perchlorate ion pair could not be concentrated by evaporation to dryness at 60°C as they appeared to form volatile derivatives since no degradative products could be detected. The sulphonate ion pair was selected as it produced higher recovery (81% at 5 μg ml^{-1} drug plasma concentration) than did the tetrabutylammonium hydrogen sulphate (74%) when both were compared at a concentration of 0.01 *M* in 90% methanol. The paired-sulphonate eluates could also be concentrated 5—10 times by evaporation to dryness at 60°C without any loss. This concentration step permitted the determination of the diminazene level (0.042 μg ml^{-1}) in goat plasma 2 weeks after an intravenous dose of 2 mg kg^{-1}.

However, recovery at submicrogram concentrations was lower than that at higher concentrations (77.3% at 0.05 μg ml^{-1} compared with 102.7% at 5 μg ml^{-1}). This is attributable to dissociation of the ion pair, which occurs typically at low concentrations [14]. However, the system is still sensitive and flexible enough as the recovery was increased from 40% at 0.05 μg ml^{-1} concentration to 77% by increasing the concentration of the ion pair from 0.01 to 0.025 *M*.

CONCLUSION

The procedure described provides a rapid and sensitive method for quantitating intact diminazene in biological fluids and in pharmaceutical preparations. The extraction step using ion-pair extraction on Sep-Pak C_{18} is short and simple and eliminates the need to split the diminazene molecule. Recovery from plasma is high (92%) and reproducible (mean assay coefficient of variation is 6.5%). Heptanesulphonic acid, perchloric acid—sodium perchlorate or tetrabutylammonium hydrogen sulphate may be used as pairing ions to effect extraction with a solvent with moderate solvating ability. The limit of quantitation in plasma is 50 ng ml^{-1}. The method has been used to follow diminazene levels in goat plasma, whole blood, milk and urine, for 2 weeks after an intravenous dose of 2.0 mg kg^{-1} body weight.

ACKNOWLEDGEMENTS

One of us (Y.O.A.) thanks the entire staff of the Department for the tremendous cooperation he has enjoyed, Drs. Sverre Weberg Teigen and Janneche Utne Skaare for their valuable contribution, and the Vice-Chancellor of Ahmadu Bello University, Professor Ango Abdullahi, for granting him sabbatical leave. The project was supported by the Norwegian Agency for International Development (NORAD) and the Department of Pharmacology and Toxicology.

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