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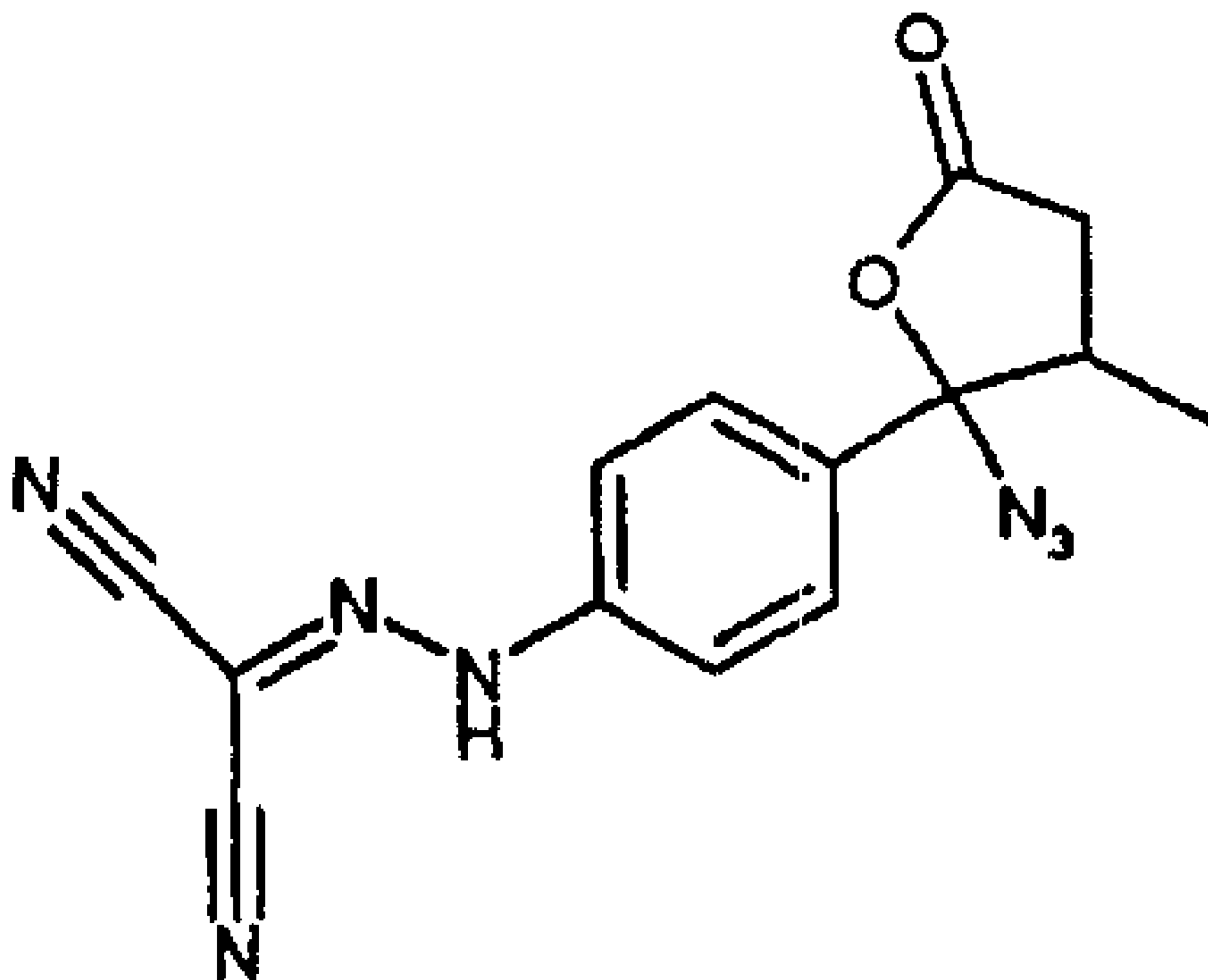
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(54) Titre : COMPOSE DE REFERENCE DESTINE A ETRE UTILISE DANS L'ANALYSE DE LOTS DE LEVOSIMENDAN
(54) Title: A REFERENCE COMPOUND FOR USE IN THE ANALYSIS OF LEVOSIMENDAN BATCHES



(I)

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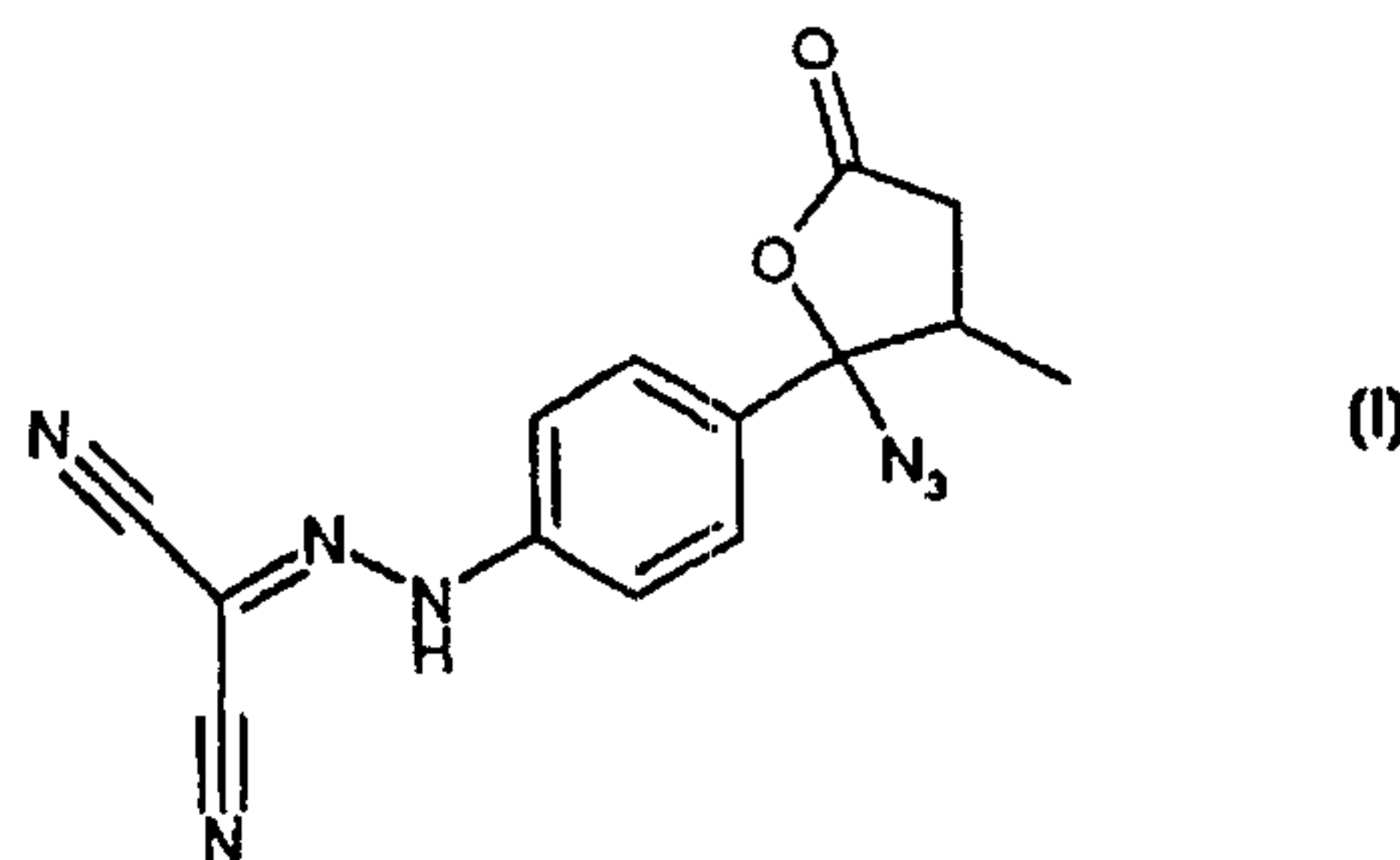
The present invention relates to a pyridazinyl derivative (I) and its use as a reference compound in the determination of potentially genotoxic impurities in levosimendan samples. The invention also relates to an analytic method for the determination of potentially genotoxic impurities in levosimendan samples wherein (I) is used as a reference compound. Levosimendan is a medicament useful in the treatment of heart failure.



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(21) International Application Number: PCT/FI99/00539 (22) International Filing Date: 18 June 1999 (18.06.99) (30) Priority Data: 981428 18 June 1998 (18.06.98) FI (71) Applicant (for all designated States except US): ORION CORPORATION [FI/FI]; Orionintie 1, FIN-02200 Espoo (FI). (72) Inventors; and (75) Inventors/Applicants (for US only): BÄCKSTRÖM, Reijo [FI/FI]; Poutamäentie 14 F 68, FIN-00360 Helsinki (FI). HEINONEN, Tuula [FI/FI]; Aamuyöntie 15 B, FIN-02210 Espoo (FI). HAUTA-AHO, Tuula [FI/FI]; Hirvikuja 3, FIN-01450 Vantaa (FI). (74) Agent: ORION CORPORATION; Orion Pharma, Industrial Property Rights, P.O. Box 65, FIN-02101 Espoo (FI).		(81) Designated States: AE, AU, BA, BG, BR, CA, CN, CZ, EE, GE, HR, HU, ID, IL, IN, IS, JP, KP, KR, LT, LV, MK, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TR, UA, US, UZ, YU, ZA, Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 27 January 2000 (27.01.00)

(54) Title: A REFERENCE COMPOUND FOR USE IN THE ANALYSIS OF LEVOSIMENDAN BATCHES**(57) Abstract**

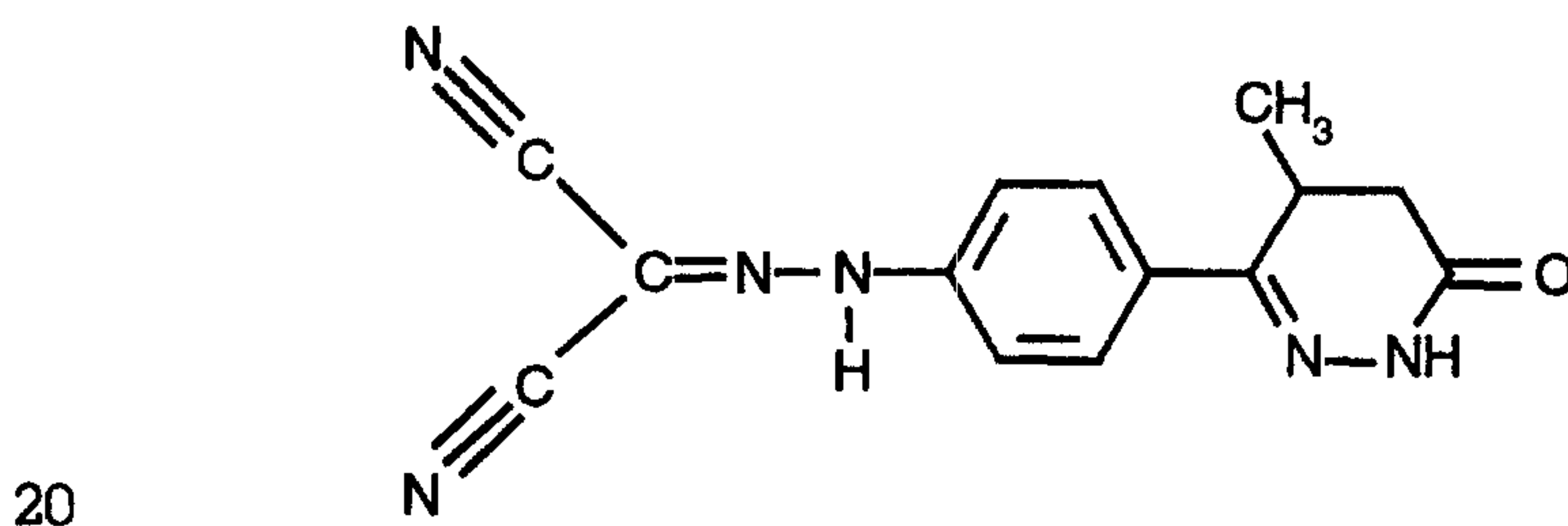
The present invention relates to a pyridazinyl derivative (I) and its use as a reference compound in the determination of potentially genotoxic impurities in levosimendan samples. The invention also relates to an analytic method for the determination of potentially genotoxic impurities in levosimendan samples wherein (I) is used as a reference compound. Levosimendan is a medicament useful in the treatment of heart failure.

A REFERENCE COMPOUND FOR USE IN THE ANALYSIS OF LEVOSIMENDAN BATCHES

Background of the invention

The present invention relates to [[4-(2-azido-3-methyl-5-oxotetrahydrofuran-2-yl)phenyl]hydrazono]propanedinitrile (I) and its use as a reference compound in the analysis of batches of levosimendan synthesis, particularly in the determination of potentially genotoxic impurities in samples of a levosimendan batch. The present invention also relates to an analytic method for the determination of potentially genotoxic impurities in samples of a levosimendan batch wherein [[4-(2-azido-3-methyl-5-oxotetrahydrofuran-2-yl)phenyl]hydrazono]propanedinitrile (I) is used as a reference compound.

Levosimendan, which is the (-)-enantiomer of [[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono]propanedinitrile, and the method for its preparation is described e.g. in EP 565546 B1. Levosimendan is potent in the treatment of heart failure and has significant calcium dependent binding to troponin. The use of levosimendan in the treatment of myocardial ischemia is described in WO 93/21921. The hemodynamic effects of levosimendan in man are described in Sundberg, S. et al., Am. J. Cardiol., 1995; 75: 1061-1066. Levosimendan is represented by the formula:

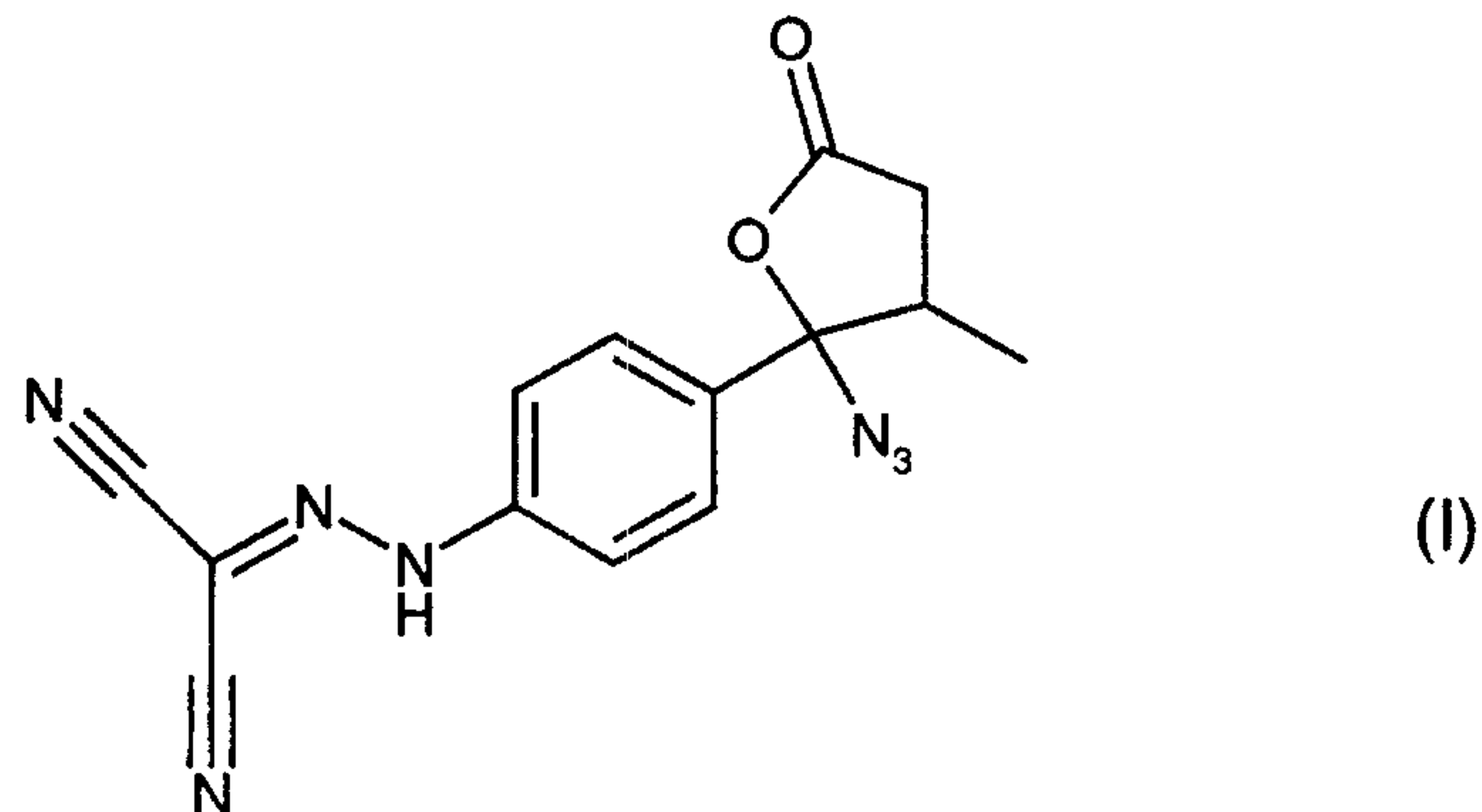


Clinical studies have confirmed the beneficial effects of levosimendan in heart failure patients.

Levosimendan can be prepared in high purity and in nearly quantitative yields by treating (-)-6-(4-aminophenyl)-5-methyl-4,5-dihydro-3(2H)-pyridazinone with sodium nitrite and malononitrile as described in EP 565546 B1. However, it has been found that samples of levosimendan as synthesized show potential genotoxic properties occasionally in the bacterial mutagenity test (Ames test).

Summary of the invention

It has now been found that the potential genotoxic properties of levosimendan samples are caused by an azido derivative impurity of formula (I)



5 This azido derivative is a potent mutagen which can be formed during levosimendan synthesis in an amount sufficient to give a positive result in the bacterial mutagenity test. This compound named [[4-(2-azido-3-methyl-5-oxotetrahydrofuran-2-yl)phenyl]hydrazono]propanedinitrile (I) is therefore useful as a reference compound in the routine analysis of batches of levosimendan synthesis,
 10 particularly in the determination of potentially genotoxic impurities in levosimendan batches. The presence of (I) in a levosimendan batch indicates that further recrystallization is necessary for obtaining levosimendan material suitable for use as a medicine.

Brief description of the drawings

15 FIG. 1 is a HPLC-chromatogram of the [[4-(2-azido-3-methyl-5-oxotetrahydrofuran-2-yl)phenyl]hydrazono]propanedinitrile standard (0.4 µg/ml).

FIG. 2 is a HPLC-chromatogram of levosimendan raw material.

Detailed description of the invention

20 The present invention provides a reference compound having structure (I) for the determination of potentially genotoxic impurities in samples of a levosimendan batch. The present invention also provides a reference preparation for the determination of potentially genotoxic impurities in samples of a levosimendan batch comprising compound (I) optionally together with an analytically acceptable carrier, and in particular essentially in the absence of pyridazinyl derivatives.

The present invention also provides the use of (I) as a reference compound in the determination of potentially genotoxic impurities in samples of a levosimendan batch.

5 The present invention also provides an analytic method for the determination of potentially genotoxic impurities in samples of a levosimendan batch characterized in that compound (I) is used as a reference compound, particularly as said levosimendan sample is analyzed by High Pressure Liquid Chromatography (HPLC).

10 Furthermore, the present invention provides an analytic method for the determination of potentially genotoxic impurities in samples of a levosimendan batch which method comprises preparing a reference standard solution by dissolving compound (I) in solution, preparing a test solution by dissolving a sample of a levosimendan batch in solution, obtaining a HPLC chromatogram of said reference standard solution, obtaining a HPLC chromatogram of said test solution and determining the concentration of compound (I) in the sample.

15 The term "analytically acceptable carrier" means here a carrier which does not hamper the qualitative or quantitative analysis of (I). The selection of a suitable analytically acceptable carrier is well known to one skilled in the art. An example of an analytically acceptable carrier is dimethyl sulphoxide.

20 Compound (I) can be prepared by treating 6-(4-aminophenyl)-5-methyl-4,5-dihydro-3(2H)-pyridazinone with sodium nitrite and malononitrile wherein sodium nitrite is used in molar excess and separating (I) from the reaction mixture. The procedure is described in detail in Example 1.

The product of a levosimendan batch is preferably analyzed using High Pressure Liquid Chromatography (HPLC). Suitable apparatus include e.g. C-8
25 reversed phase HPLC column with UV-detection at 360 nm. The mobile phase is e.g. a mixture of phosphate buffer pH 2.1 and acetonitrile. The levosimendan sample is dissolved in suitable solvent such as a mixture of dimethyl sulfoxide, methanol and water. The reference solutions are prepared by dissolving compound (I) in suitable solvent. The retention times of levosimendan and compound (I) or diastereomers
30 thereof are determined in the chromatographic conditions used. The amount of (I) in the levosimendan sample is determined by comparing the chromatograms obtained for the sample solution and the reference solution. The procedure is described in detail in Example 2.

The amount of the undesired impurity (I) in a levosimendan batch can be reduced by methods known in the art such as recrystallization. Recrystallization of levosimendan can be performed from any suitable solvent acetone being the preferred solvent.

5 EXAMPLES

Example 1. Preparation of [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propanedinitrile (mixture of diastereomers)

6-(4-Aminophenyl)-4,5-dihydro-5-methyl-3(2H)-pyridazinone (153 g, 0.75 mol) was dissolved in acetic acid (750 mL). Solid sodium nitrite (210 g, 3.0 mol) was slowly added to the solution at 10 - 20 °C. After the addition the mixture was stirred for 90 min at 10 - 20 °C. The reaction mixture was poured rapidly to a 20 °C solution of malononitrile (150 g, 2.3 mol) and water (1500 mL). The resulting mixture was stirred at 20-25 °C for 60 min and then filtered and washed with water. The wet solid was extracted successively with tetrahydrofuran (300 mL) and ethyl acetate (1900 mL). The combined extracts were dried with sodium sulphate and the solvents evaporated under vacuum. The residue was triturated with ethyl acetate (200 mL), filtered and the filtrate was evaporated under vacuum. The residue (8.0 g) was purified chromatographically on silica gel using toluene-ethyl acetate 2:1 as the eluent. After crystallization from toluene the yield of pure mixture of diastereomers was 1.0 g. The major component of the mixture can be enriched by multiple crystallizations from toluene, but for analytical purposes the mixture is adequate. ¹H-NMR (400MHz, DMSO-d₆, σ) major diastereomer 1.06 (d, 3H, J=6Hz), 2.55 (dd, 1H, J=16 Hz, 11 Hz), 2.67 (m, 1H), 2.78 (dd, 1H, J=16 Hz, 8 Hz), 7.59 (d, 1H, J= 9Hz), 7.62 (d, 1H, J= 9Hz), 12.6 (broad s, 1H), minor diastereomer 0.60 (d, 3H, J=7 Hz), 2.43 (dd, 1H, J=18 Hz, 2 Hz), 2.78 (m, 1H), 3.14 (dd, 1H, J=8 Hz, 18 Hz), 7.54 (d, 1H, J=9 Hz), 7.61 (d, 1H, J=9 Hz), 12.6 (broad s, 1H).

Example 2. Use of [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propanedinitrile (I) as a reference compound in HPLC-chromatography

The HPLC (High Pressure Liquid Chromatography) method for analysis of compound (I) in levosimendan raw material was based on the C-8 reversed phase HPLC column with UV-detection at 360 nm. The mobile phase consisted of acetonitrile and phosphate buffer pH 2.1.

Reagents:

1. Methanol, HPLC grade
2. Sodium dihydrogen phosphate, $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$, Merck
3. Ortho phosphoric acid, H_3PO_4 , Merck
- 5 4. Phosphate buffer pH 2.1:
Dissolve 1.8 g of sodium dihydrogen phosphate (NaH_2PO_4) in water and add 2.0 ml of phosphoric acid. Adjust the pH if necessary with 2 M sodium hydroxide or 1 M phosphoric acid. Dilute to 1000.0 ml with water.
5. Dimethyl sulphoxide, BDH
- 10 6. Acetonitrile, HPLC grade
7. Solvent: Mix 200 ml of methanol with 800 ml of water
8. [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]-propanedinitrile reference standard
- 15 Standard solutions (reference preparations of compound (I)):

Stock solution: 10.00 mg of [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propanedinitrile is dissolved in a 100 ml volumetric flask in 20 ml of dimethyl sulphoxide and filled to volume with methanol.
- 20 Standard solution 1 (50 $\mu\text{g}/\text{ml}$): 5.00 ml of the stock solution is diluted to 10.0 ml with solvent.
Standard solution 2 (0.4 $\mu\text{g}/\text{ml}$ = 40 ppm): 1.00 ml of the stock solution is diluted to 250.0 ml with solvent.
Quantitation limit solution (0.1 $\mu\text{g}/\text{ml}$ = 10 ppm): 5.00 ml of standard solution 2 is
- 25 diluted to 20.0 ml solvent.

Test solution

- 100 mg of test sample is dissolved in a 10 ml volumetric flask in 8 ml of dimethyl sulphoxide and filled to volume with solvent.

Chromatographic conditions:

- | | |
|------------------|---|
| Apparatus | Liquid chromatograph |
| 35 Detector | UV-VIS, detection wavelength 360 nm |
| Column | Symmetry C 8, 3.0 μm , 7.5 cm x 4.6 mm |
| Oven temperature | ambient |
| Mobile phase | A: phosphate buffer pH 2.1 |

		B: acetonitrile
	% B	0 min 20 %
		5 min 30 %
		15 min 30 %
5		30 min 90 %
	Flow rate	0.8 ml/min
	Injection volume	100 µl
	Run time	30 min
	Post time	10 min
10	Retention times:	levosimendan about 13 min
		Compound (I) minor diastereomer: about 24 min
		Compound (I) major diastereomer: about 25 min

System suitability

- 15 1. Replicate injections of quantitation limit solutions must be repeatable;
6 injections, RSD = 10.0%.

Procedure

- 20 After the system suitability criteria are met, proceed with the standard and sample
injections.

Calculations

- 25 The concentration of the major diastereomer of compound (I) in ppm will be
calculated according to the following equation:

$$\frac{C_{st} \cdot R_x \cdot 10 \cdot 1000000}{R_{st} \cdot w}$$

C_{st} = concentration of the major diastereomer of compound (I) in [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propane-dinitrile standard solution (mg/ml)

- 30 R_x = the peak area of the major diastereomer of compound (I) in the chromatogram of the test solution

R_{st} = the peak area of the major diastereomer of compound (I) in the chromatogram of the [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)-phenyl]hydrazono]propanedinitrile standard solution

- 35 w = weight of the sample

10 = dilution factor of the sample (ml)

The concentration of the minor diastereomer of compound (I) in ppm will be calculated according to the following equation. The major diastereomer of compound (I) is used as a reference standard.

$$5 \quad \frac{C_{st} \cdot R_x \cdot 10 \cdot 1000000}{R_{st} \cdot w} \cdot 1.023$$

C_{st} = concentration of the minor diastereomer of compound (I) in [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propane-dinitrile standard solution (mg/ml)

10 R_x = the peak area of the minor diastereomer of compound (I) in the chromatogram of the test solution

R_{st} = the peak area of the minor diastereomer of compound (I) in the chromatogram of the [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)-phenyl]hydrazono]propanedinitrile standard solution

w = weight of the sample

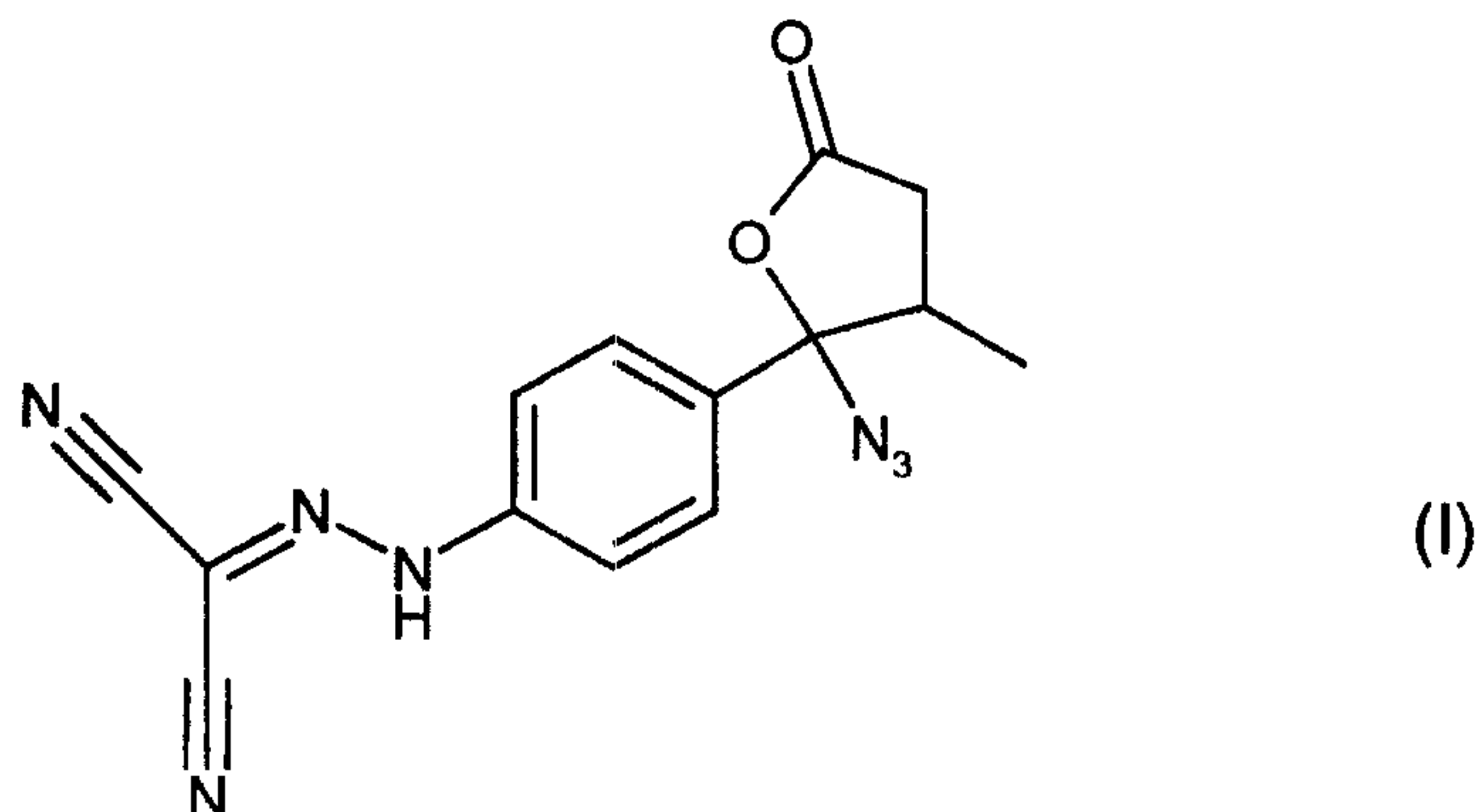
15 10 = dilution factor of the sample (ml)

1.023 = correction factor for the minor diastereomer of compound (I)

The chromatogram of the [[4-(2-azido-3-methyl-5-oxotetrahydro-furan-2-yl)phenyl]hydrazono]propanedinitrile standard (0.4 µg/ml) obtained is shown in Figure 1. The retention time for the major diastereomer of compound (I) is 25.180 min and for the minor diastereomer of compound (I) 24.866 min. The chromatogram of levosimendan raw material is shown in Figure 2.

CLAIMS

1. A compound characterized by having the structure of formula I



5 and diastereomers thereof.

2. A reference preparation for the determination of potentially genotoxic impurities in samples of a levosimendan batch comprising compound of claim 1.

3. A preparation according to claim 2 essentially in the absence of pyridazinyl compounds.

10 4. A reference preparation for the determination of potentially genotoxic impurities in samples of a levosimendan batch comprising compound of claim 1 together with an analytically acceptable carrier.

5. A preparation according to claim 4 essentially in the absence of pyridazinyl compounds.

15 6. Use of the compound of claim 1 as a reference compound in the determination of potentially genotoxic impurities in samples of a levosimendan batch.

20 7. An analytic method for the determination of potentially genotoxic impurities in samples of a levosimendan batch characterized in that compound of claim 1 is used as a reference compound.

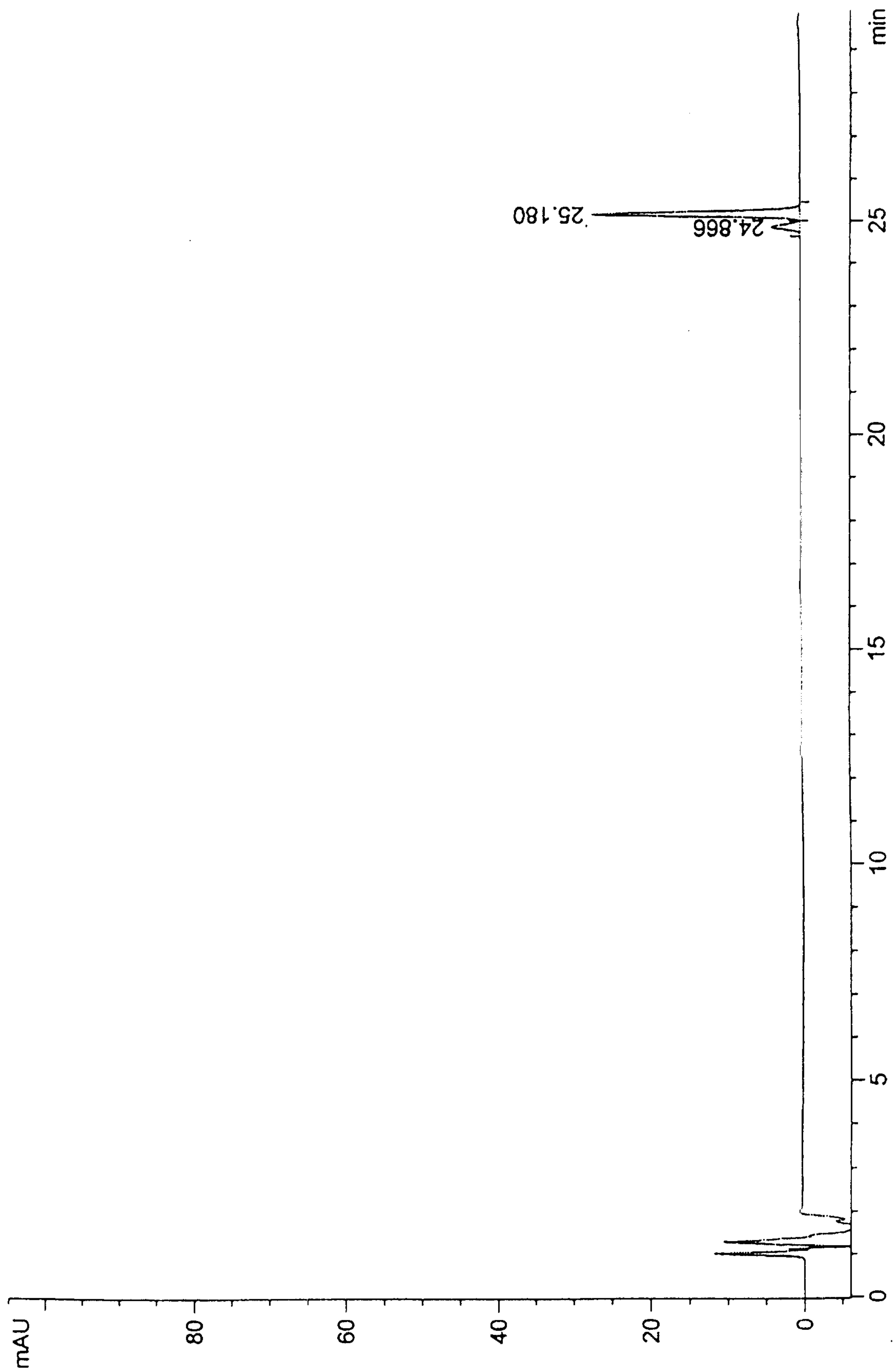
8. A method according to claim 7 characterized in that a sample of a levosimendan batch is analyzed by High Pressure Liquid Chromatography (HPLC).

9. An analytic method for the determination of potentially genotoxic impurities in samples of a levosimendan batch which method comprises preparing a reference standard solution by dissolving compound of claim 1 in solution, preparing a test solution by dissolving a sample of a levosimendan batch in solution, obtaining
- 5 a HPLC chromatogram of said reference standard solution, obtaining a HPLC chromatogram of said test solution and determining the concentration of compound of claim 1 in the sample.

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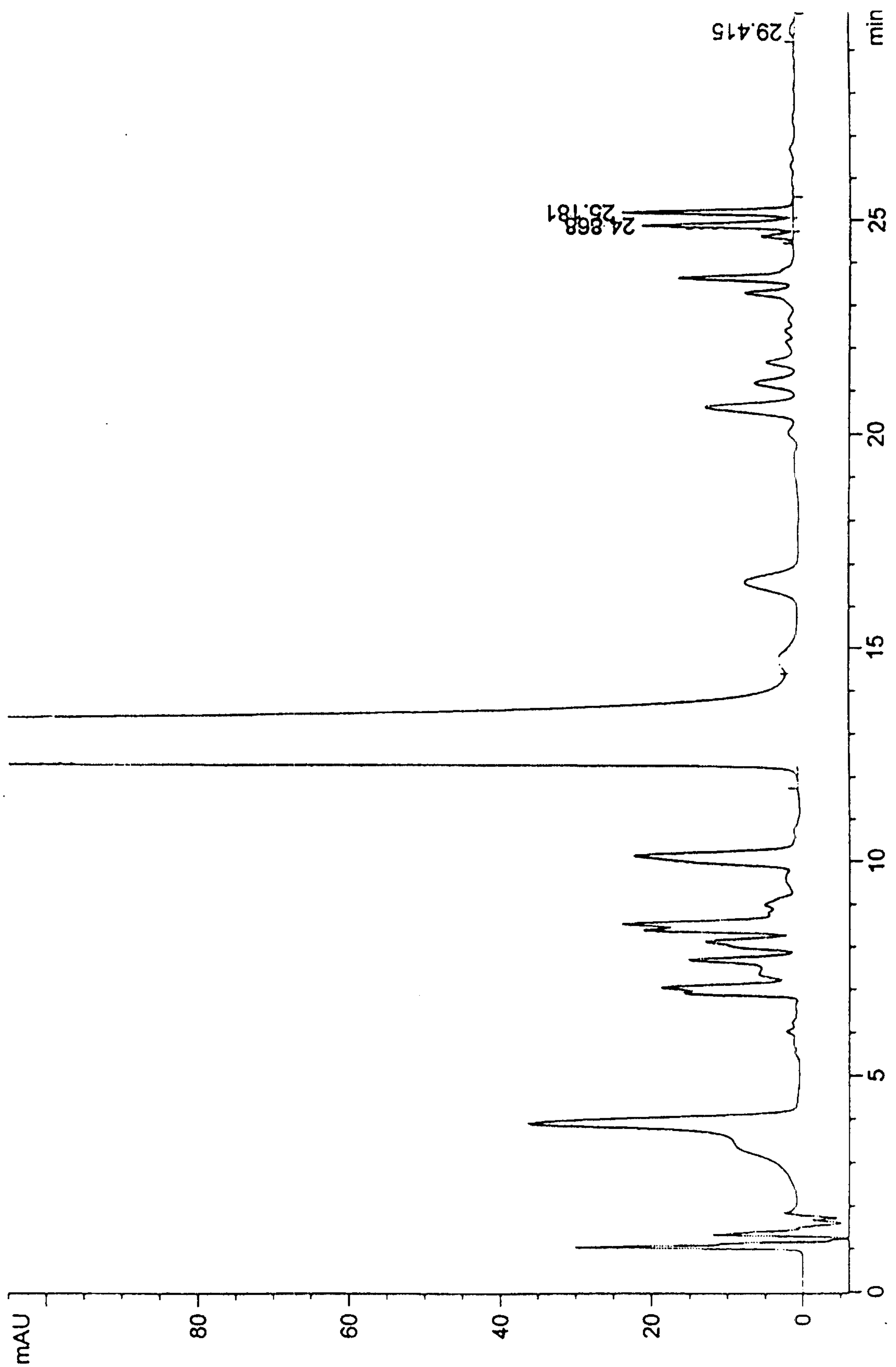
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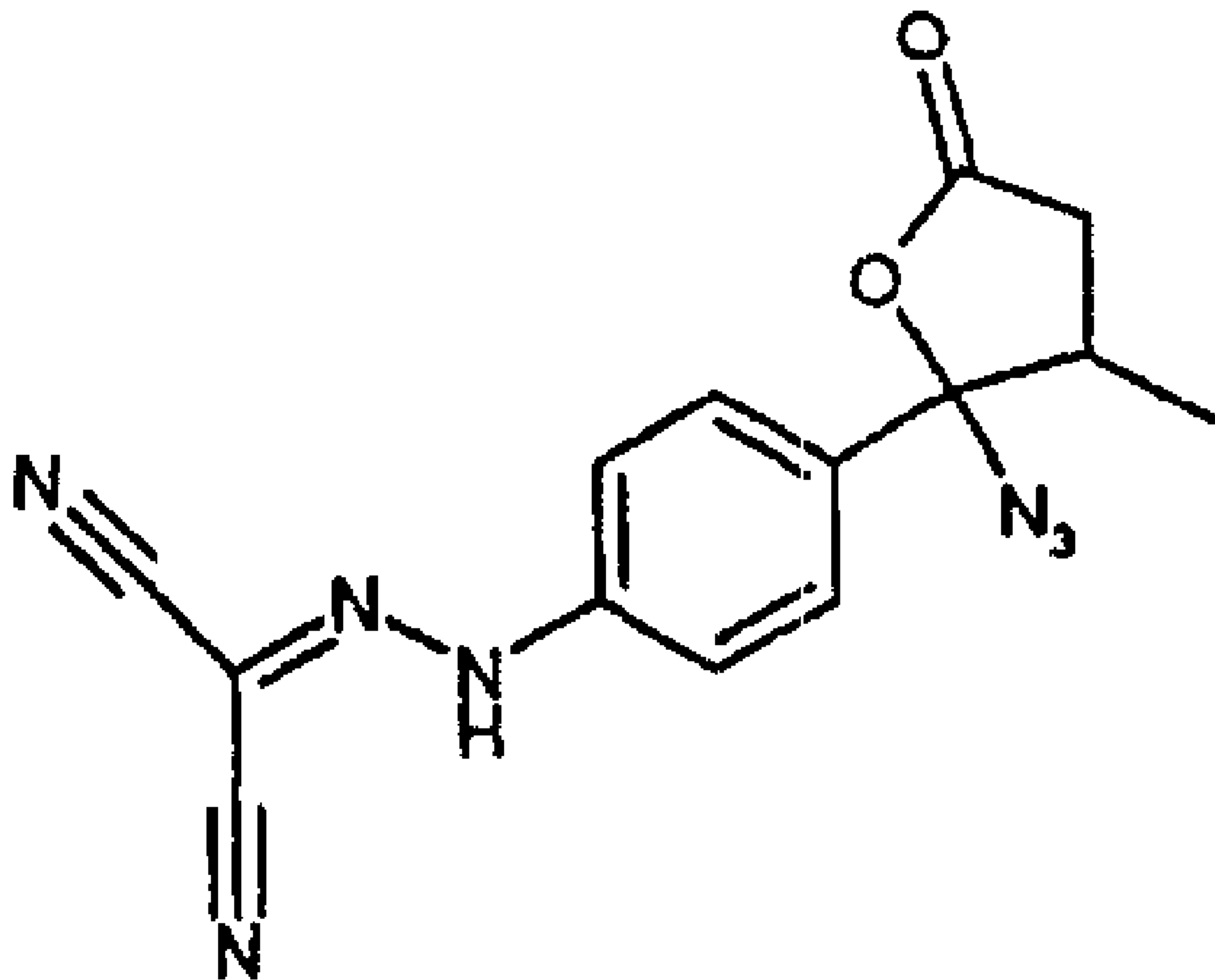
*FIG. 1*

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**FIG. 2**



(1)