A Note on
QUANTITATIVE ANALYSIS OF FLESINOXAN IN PLASMA AND URINE
AT THE pg/ml LEVEL USING GC-ECD

M.P. van Berkel, H. de Bree and K. Sierat

Duphar Research Laboratories, P.O. Box 2
1380 AA Weesp, The Netherlands

Ceelan et al. [1] developed an HPLC method for the determination
in plasma of flesinoxan (given as the hydrochloride; DU 23973), a
relatively large and polar molecule of low volatility, shown on left
in the diagram below. However, on the basis of the results of the
first safety and tolerance study the dose was lowered to 0.25 mg/person,
resulting in plasma levels in the pg/ml range. For the pharmacokinetic
monitoring of the drug at this low dose, the HPLC method was unsuitable.
A new analytical procedure had to be developed using a completely
different strategy aiming at utmost sensitivity. GC-ECD* is a highly
sensitive technique which could be suitable after hydrolysis of the
drug to the FBz and then esterification with PFB(B):

Materials.— Flesinoxan and the i.s. (Fig. 1) were obtained from
our own laboratories; both were of >98% purity. From 3- and 4-FBz
(Merck, FRG; 821795 and 818537) the PFB esters were prepared according

* ECD denotes electron-capture detector; FBz, fluorobenzoic acid or
benzoate; MIBK, methyl isobutyl ketone; PFB(B), pentafluorobenzyl
(bromide); d.f., degrees of freedom; i.s., internal standard. 'Ether'
is diethyl ether. Drug and i.s. concentrations are for hydrochloride.
Fig. 1. The internal standard (i.s.), DU 122049.

\[ \text{CH}_2\text{OH} \]
\[ \text{O} \]
\[ \text{O} \]
\[ \text{H} \]
\[ \text{N} \]
\[ \text{H} \]
\[ \text{H} \]
\[ \text{N} \text{CH}_2\text{CH}_2\text{NH-C} \]
\[ \text{F} \]
\[ .2\text{HCL} \]

To Knapp [2]; after additional purification each was shown by GC-ECD to be >99% pure. All organic solvents were distilled twice using a 2-m vigreux in an all-glass apparatus. All chemicals were of analytical grade. Tripropylamine (Fulka, Switzerland; 93240) was purified by filtration through activated silica. Aqueous solutions were made in double-distilled water and washed 3 times with iso-octane.

**Stock solutions** containing ~10 mg flesinoxan or i.s. in 100 ml methanol were prepared every 3 months, and used as freshly prepared x1000 dilutions in appropriate solvents. For 3-FBz and 4-FBz as used for optimization of some of the steps, a 10 mg/100 ml stock solution in diethyl ether was freshly diluted x1000 with methanol prior to use. The esters as also used to check the derivatization, clean-up and GC procedure were dissolved (10 mg/ml) in and diluted x100 with iso-octane. All stock solutions were stored at 4°C.

**Sample preparation.**- To 5 ml plasma or urine, 10 ng of i.s. and 1 ml 25% (w/w) ammonia were added, and the mixture extracted with 5 ml MIBK. The organic phase was washed with 2.5 ml 1 M NaOH. If the expected concentration was <500 pg/ml, ~10 ml plasma or urine was taken, and 2 ml ammonia and 10 ml MIBK were used.

**Hydrolysis and clean-up.**- The MIBK phase was evaporated to dryness, 200 μl 15% (w/v) HCl was added, and the vials were closed tightly and kept at 110°C for 16 h. The samples, after cooling and adding 300 μl 5 M NaOH, were washed with 2 ml ether/n-pentane (4:1 by vol.). The aqueous phase was acidified with 400 μl 15% HCl and extracted with 2 ml ether/n-pentane. The resulting organic phase was washed with 200 μl water.

**Preparation of derivatives.**- The organic phase was evaporated down to ~400 μl at room temperature with a gentle stream of N₂, transferred to a 500 μl conical vial, and taken gently to dryness with N₂. The residue was dissolved in 50 μl of a freshly prepared derivatization reagent: 400 μl tripropylamine, 200 μl PFBB (Pierce, U.S.A.; 58220) and 2 ml acetone. After 15 min (room temp.) 10 μl 100% ethylamine (Fluka; 02940) was added to react with the excess of PFBB (room temp., 60 min). The mixture was acidified with 50 μl 35% (w/v,aq.) perchloric acid and extracted with 50 μl iso-octane/ether.

**HPLC clean-up.**- The organic phase was injected completely, using a valve (Rheodyne, U.S.A.; 7125) with a 50 μl sample loop, onto a