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A reversed-phase high-performance liquid chromatographic method for the determination of Clanfenur in rat and human plasma

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Abstract: A selective and specific high-pressure liquid chromatographic (HPLC) method for the simultaneous assay of Clanfenur and its metabolites in biological fluids of interest has been developed which is suitable for routine analysis, using micro volumes (0.1 ml) of plasma samples only. After protein precipitation the extract is analysed by reversed-phase HPLC with UV detection. Excellent recovery, linearity, accuracy and precision (less than 5% for plasma) are achieved by the assay which is able to quantify Clanfenur and its metabolites in plasma at concentrations between 0.025 and 5.0 mg l^{-1}.

Keywords: *Clanfenur; reversed-phase HPLC; benzoylphenyl ureas; antineoplastic drugs.*

Introduction

Substituted benzoylphenylureas have been identified as a novel class of orally and intraperitoneally active antineoplastic agents with haemopoiesis-stimulating properties considered useful in the treatment of human solid tumours [1–5]. The compound Clanfenur [1-(2-fluoro-6-dimethyl-aminobenzoyl)-3-(4-chlorophenyl)urea] was selected in this series because of this dualistic effect and its interesting toxicological profile, including lack of mutagenicity [6], absence of cytotoxicity in chicken embryonic fibroblasts, absence of cross-resistance in cell lines selected for multiple drug resistance [7] and activity in carcinomas, among which human colon tumour xenografts [1]. Its chemical structure is shown in Fig. 1. The low solubility of Clanfenur in water (0.4 mg l^{-1}) and saline (0.25 mg l^{-1}) can raise problems to achieve target plasma levels which should be pursued for optimal therapeutic efficacy after absorption from the gastrointestinal tract into the circulation. Furthermore, the parent drug is rapidly metabolized by liver enzymes to several metabolites. On the other hand, it was found that the drug is highly protein-bound (>99%). As a consequence the relationship between serum Clanfenur and/or its metabolite concentration and Clanfenur's therapeutic efficacy and toxicity has to be established.

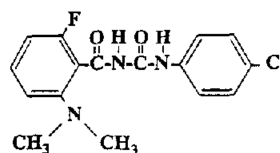

Figure 1
Structure of Clanfenur.

A sensitive and specific assay for this compound in biological fluids is a key requirement in order to monitor the time course of absorption and elimination of Clanfenur, to define its pharmacokinetics and to help in testing of new formulations. Determination of benzoylphenylureas by HPLC methods has been reported [8]. The analysis of Clanfenur carried out previously required isolation of components using solid-phase extraction onto C_{18} bonded silica and subsequent liquid–solid chromatography using a silica gel HPLC column and isocratic elution (Solvay–Duphar B.V., unpublished). The assay was sensitive

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enough to measure with precision and accuracy 0.25 mg l^{-1} Clanfenur in plasma. However, for routine requirements this method was time- and plasma-consuming, interferences of metabolites or other structural analogues with Clanfenur were found and the compound may be irreversibly bound to the HPLC support. Therefore, the objectives of the present work were to develop an analytical method for Clanfenur in plasma by single liquid–liquid extraction followed by gradient elution on a reversed-phase HPLC support. The method described below offered the advantage that the total analysis time was reduced and the overall resolution per unit time was increased.

Experimental

Reagents and materials

Analytical grade chemicals were used throughout this study with the following exceptions: methanol and acetonitrile were HPLC grade (Merck, Darmstadt, Germany). The water used was prepared by a Milli-Q deionization unit (Millipore, Bedford, MA, USA) and stored in glass containers for a maximum of 1 week, then discarded. All solvents and solutions for HPLC were filtered through 0.45 μm membrane filters (Millipore type RA and FH for aqueous and organic solvents, respectively). Prior to use, the components of the solvent system were mixed and degassed under reduced pressure, and a helium sparge was maintained through the solvent for the duration of the chromatographic analysis.

To prepare the mobile phase 7.88 g of ammonium formate (97%) is weighed into a large beaker containing 2500 ml of water and the pH of the solution is adjusted to 6.45 by the addition of 1.0 M sodium hydroxide with thorough mixing. Then 300 ml of the ammonium formate buffer (0.05 M, pH 6.45) is transferred to a 1000 ml graduated cylinder and diluted to volume with acetonitrile.

Supplies of Clanfenur (Charge CAG 03486A) and Diflubenzuron [1-(2,6-difluorobenzoyl)-3-(4-chlorophenyl)urea] (Charge ARS 85730N), the internal standard, were obtained from the Section of Analysis Pharma, Solvay–Duphar Research B.V.(Weesp, The Netherlands) and were used without further purification. A stock solution of Clanfenur and Diflubenzuron was prepared by accurately weighing 5.0 mg into a 100.0 ml volumetric flask containing 25.0 ml methanol, which was ultrasonicated for 10 min, removed, and made up to volume with methanol. Both stock solutions were stable for at least 4 weeks provided they were maintained at +4°C when not in use and not exposed to direct sunlight. Working solutions over the range 0.5–100.0 mg ml^{-1}, necessary to construct a daily calibration curve, were freshly prepared starting with serial dilution of the stock solution in methanol.

Plasma samples

Normal human plasma (60.7 g l^{-1} protein) pooled from a panel of healthy subjects was obtained from the Central Blood Bank (Amsterdam, The Netherlands). Drug-free blood was withdrawn from rats via a cannula in the carotid artery into heparinized tubes, mixed and centrifuged. The blank plasma (63.4 g l^{-1} protein) obtained was transferred to plain tubes, which were kept at −80°C prior to analysis. Fetal calf serum (54.5 g l^{-1} protein) was obtained from Gibco Europe (Paisley, UK).

Extraction of plasma samples

Plasma samples for the assay of Clanfenur were first thawed within 30 min at ambient temperature (benchtop) and then centrifuged at 2000*g* for 5 min to remove any fibrous material. Plasma (0.1 ml) was transferred to a 1.5 ml polypropylene Eppendorf centrifuge tube. Then 150 ng (0.1 ml methanol) internal standard and acetonitrile (0.2 ml) were added and mixed by vortex. The internal standard solution is used to quantify the parent drug. Subsequently, an additional volume of 0.4 ml of acetonitrile was added and mixed by vortex. The samples were centrifuged at 15,000*g* for 15 min. The resulting supernatant transferred to a glass conical tube and evaporated to dryness at 40°C under a stream of nitrogen. The residues were reconstituted in 0.4 ml of 0.05 M ammonium formate buffer (pH 6.45) containing 20% (v/v) acetonitrile by mixing on a vortex mixer for 1 min.

Apparatus

The HPLC system comprised two Model 6000 solvent delivery pumps equipped with an automated gradient controller (Waters Associates, Milford, MA, USA) and connected to a Rheodyne syringe loading sample injector model 7010 with a 100-μl loop or an LC 600

autosampler with a 100-μl loop (Perkin–Elmer, Norwalk, CT, USA). The 100 × 4.6 mm i.d. analytical column was packed with 3-μm MicroSphere C_{18} and coupled with a 10 mm Chromguard column (Chrompack, Middelburg, The Netherlands). The analytes were monitored continuously by a Model 440 UV detector (Waters) at 254 nm. The signals from the detector were evaluated with the system software OMEGA (Perkin–Elmer).

Chromatography

The solvent system used consisted of a linear gradient elution of 0.05 M ammonium formate buffer, pH 6.45 (solvent A) and acetonitrile containing 30% (v/v) ammonium formate buffer (0.05 M, pH 6.45) (solvent B). The gradient profile adopted was: t = 0 min, 20% B; t = 1 min, 20% B; t = 15 min, 85% B; t = 17 min, 85% B; t = 18 min, 20% B; t = 20 min, 20% B at a flow rate of 1.45 ml min^{-1} at ambient temperature.

Calibration curve

At the beginning of any study a calibration curve was constructed using 0.1 ml blank plasma aliquots that had been spiked with Clanfenur in a constant addition volume of methanol over the concentration range of 0.025–5.0 mg l^{-1}; this consisted of 27 points covering nine concentrations; three replicates were assayed for each concentration. A set of quality control fetal calf serum samples spiked with Clanfenur (0.05–10.0 mg l^{-1}) was obtained from the Section of Analysis Pharma, Solvay–Duphar Research B.V. (Weesp, The Netherlands) and kept in amber coloured glassware at −80°C. These seeded control samples were interspersed within the batch of standards for sample workup. The biological content both of seeded controls and standard samples was not less than 95%.

Evaluation of the HPLC method

Quantitation. Quantitation was based on peak height ratio analyte–Diflubenzuron in the relevant concentration range of Clanfenur. Calibration curves consisting of at least nine concentrations in triplicate and covering a range of 0.025–5.0 mg l^{-1} plasma were analysed on several separate occasions. The slopes, intercepts and correlation coefficients were calculated by curve fitting using the linear approach of the type: $y = ax + b$ and compared for linearity and reproducibility. Sample concentrations were estimated by inverse prediction.

Specificity. Blank plasma samples of different species (human, rat and foetal calf serum) were processed and chromatographed, and the detection output at the retention time for Clanfenur and Diflubenzuron was recorded.

Limit of detection (LOD). Clanfenur was added to rat and human plasma (0.0025–0.1 mg l^{-1}) samples. These samples, as well as drug-free blank samples were processed and analysed. The peak heights at the retention time of Clanfenur in each blank and test sample were recorded and subjected to a paired Student's t-test.

Limit of quantitation (LOQ). Clanfenur was added to rat and human plasma (0.0025–0.1 mg l^{-1}) samples. These samples, as well as corresponding drug-free blank samples were processed, analysed and the concentrations of the drug were determined based on a calibration curve.

Accuracy and precision. Bulk plasma (0.05–10.0; mg l^{-1}) samples were prepared by someone other than the technician. Replicate samples were analysed on the day of preparation and on other days. Accuracy was expressed as the percentage of deviation of the mean observed from the nominal concentration. Precision was expressed as the mean percentage of relative standard deviation (% RSD) of the observed concentration. Between-day accuracy was calculated using the deviation of the grand mean for each concentration from the nominal concentrations. Between-day precision was calculated from the observed mean of all samples at each concentration.

Recovery. Standard plasma samples spiked with Clanfenur (0.025–5.0 mg l^{-1}) were processed and analysed. The slopes of these calibration curves were calculated as a percentage of the slopes of calibration curves prepared in acetonitrile and injected directly.

Stability in plasma. A bulk human plasma sample containing 1.0 mg l^{-1} Clanfenur was prepared and 1.0 ml samples transferred to separate tubes. One was placed directly on dry ice and the others were placed in a water bath and incubated for various periods of time at

37°C. The samples were then stored at −80°C and analysed the following day. Storage stability was evaluiated by preparing bulk plasma samples of Clanfenur (0.05–10.0 mg l^{-1}), transferring 2.0 ml samples to separate tubes and storing these samples for various periods of time at −30°C. The stability of Clanfenur was calculated as a percentage of peak height at zero-time.

Stability during HPLC analysis. A bulk human sample containing Clanfenur (1.0 mg l^{-1}) was processed and placed in autosampler vials. One set of vials was left at ambient temperature and injected in triplicate at 0, 24 and 48 h. One other set was stored at 4°C and injected at 24 and 48 h. The peak heights were compared to the zero-time peak heights.

Results and Discussion

Clanfenur is almost insoluble in water with a partition coefficient in 1-octanol–water of about 5000, but the maximum solubility in human plasma is about 60.0 mg l^{-1}. Bonded-phase chromatography on silica gel as adsorbent was selected for the analysis of Clanfenur in plasma in view of the expected hydrophobic interaction with the phase of Clanfenur and its metabolites and the advantage to increase the overall resolution per unit time by use of gradient analysis. In addition, chromatography onto C_{18} bonded silica gel in acetonitrile eliminated the irreversible adsorption of Clanfenur and interference with structural analogues observed in previous liquid–solid chromatography onto silica gel columns which increased the chromatographic efficiency and linear sample capacity. Ammonium formate was used because it is a volatile salt and showed a sufficient buffer capacity at pH 6.45. Changing the pH in the range 2.5–7.0 at 0.05 M ammonium formate buffer increased the capacity factor (k') of Clanfenur from 6.6 to 8.7, respectively. Increasing the ionic strength from 0.005 to 0.16 M ammonium formate buffer at pH 6.45 had no effect the selectivity factor (α) of Clanfenur. Based on these parameters, a mobile phase containing 0.05 M ammonium formate buffer (pH 6.45) was chosen which showed a generally good resolution and sharp peaks devoided of any tailing. The optimum gradient was selected by trial and error. The wavelength of detection was set at 254 nm, which was the maximum of the absorption recorded of both Clanfenur and the internal standard. Possible metabolites showed a maximum of absorption in the range of 248–262 nm. Furthermore, the simple and rapid procedure using a single extraction with acetonitrile yielded acceptable recoveries of Clanfenur from 0.1 ml samples of plasma. Under the experimental conditions, no influence of the high drug–protein binding on the analysis could be observed. Calibration curves (n = 6) for Clanfenur of peak-height ratio versus concentration were linear and reproducible over the calibration range (0.025–5.0 mg l^{-1}) with correlation coefficients of >0.99997, mean (±SD) slopes of 0.00075 ± 0.00002 and mean (±SD) intercepts of 0.007 ± 0.004. The limit of quantitation of the presented assay is 0.025 mg l^{-1} (% RSD = 10.5; % error = 12.7) with an absolute detection limit of 0.63 ng (signal-to-noise ratio = 2). Significant responses were seen at concentrations below 0.025 mg l^{-1} but good linearity was observed only between 0.025 and 5.0 mg l^{-1}. The overall recovery of Clanfenur from plasma was 93.7%, based on a comparison of the slope of a processed calibration curve (n = 3) to the slope of a standard curve prepared in acetonitrile and assayed without processing (n = 3). Recovery was consistent over the entire concentration range, with individual values ranging from 88.5 to 99.3%. Figure 2 shows representative chromatograms for the standard plasma solution, seeded control plasma and plasma of a rat which received an oral dosage of Clanfenur. The internal standard and Clanfenur were well resolved with retention times of about 12.8 (% RSD = 0.12) and 14.5 (% RSD = 0.17) min, respectively. There was no interference of endogenous constituents from plasma of different species (foetal calf, human and rat) at the retention times of either Clanfenur or the internal standard. Additionally, major metabolites eluted from the solid phase, respectively, at 3.4, 4.6, 6.2, 7.5, 9.0, 12.2 and 16 min without affecting the retention of the other analytes (Fig. 2). Therefore, the HPLC system appeared to have good specificity and the chromatographic analyses could be performed with sufficient reproducibility at ambient temperature. No attempt was made to determine the elution order of the metabolites since quantitation of the metabolites was not the objective here. The precision and accuracy of the assay were assessed by analysing replicate

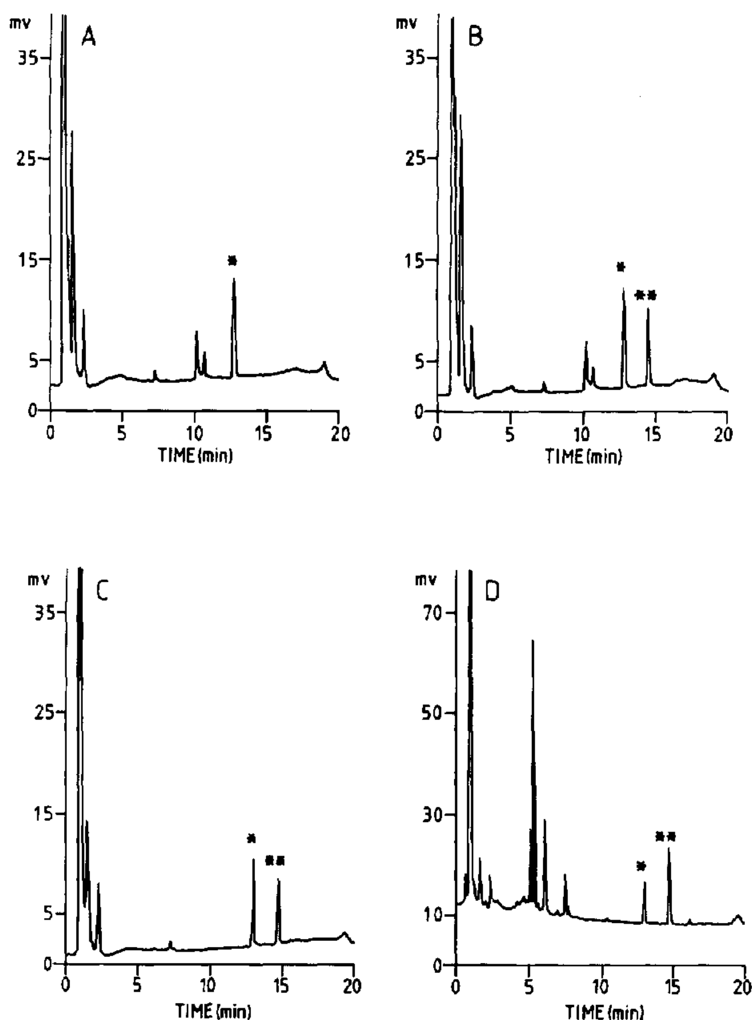

Figure 2
Typical chromatograms of standard and unknown plasma extracts. Retention times: internal standard (Diflubenzuron) 12.9 min (*), Clanfenur 14.5 min (**). (a) Extract of blank human plasma; (b) extract of standard human plasma spiked with Clanfenur (1.0 mg l^{-1}); (c) extract of control foetal calf serum spiked with Clanfenur (1.0 mg l^{-1}); (d) extract of rat plasma collected at 3.0 h after administration of an oral dosage of 2000 mg Clanfenur kg^{-1} BW (2.36 mg l^{-1}). Samples a–d were spiked with internal standard before extraction. UV detection at 254 nm with sensitivity 0.04 AUFS.

spiked samples of plasma at selected concentrations of Clanfenur over the range of 0.05–10.0 mg l^{-1} (Tables 1 and 2). The mean (±SD) within-day accuracy was 100.7 ± 2.4% at concentrations of 0.05–5.0 mg l^{-1} and the corresponding mean (±SD) precision (% RSD) was 2.2 ± 0.6%. The mean (±SD) between-day accuracy at the same concentration range was 98.5 ± 1.8% and the mean between-day precision (% RSD) was 3.5 ± 1.1%. Clanfenur (1.0 mg l^{-1}) was stable in human plasma *in vitro* for at least 24 h at 37°C. The results are summarized in Table 3. Extracts of plasma samples containing 1.0 mg l^{-1} were stable for at least 48 h at ambient temperature following reconstitution in the mobile phase, with a precision (% RSD) of <3.0% ($n = 4$). At concentrations covering a range of 0.05–10.0 mg l^{-1}, Clanfenur was stable for more than 1 year in human plasma and foetal calf serum stored at −30°C. These results indicate that plasma samples from (pre)clinical studies can be stored at −30°C and analysed within 365 days of collection to obtain valid results.

Conclusion

In conclusion, a sensitive, specific, reproducible and accurate HPLC method has been developed and validated for the bio-analysis of Clanfenur in one procedure. Additionally, the single extraction with acetonitrile requires

Table 1
Intra-day precision and accuracy of Clanfenur HPLC assay procedure

	Theoretical Clanfenur concentration (μg l^{-1})							
	25.1	50.3	100.5	251.3	502.5	1005.0	2512.5	5025.0
Samples (*n*)	4	4	4	4	4	4	4	4
Mean	22.1	50.5	105.8	244.8	503.6	1019.1	2518.9	5020.9
±SD	2.6	1.4	2.9	5.2	11.1	13.1	61.9	89.1
Precision (% RSD)	11.8	2.9	2.8	2.1	2.2	1.3	2.5	1.8
Accuracy (%)	88.0	100.4	105.3	97.4	100.2	101.4	100.3	99.9

Table 2
Inter-day precision and accuracy of Clanfenur HPLC assay procedure

	Theoretical Clanfenur concentration (μg l^{-1})								
	50	100	200	500	750	1000	2000	5000	10,000
Samples (*n*)	6	6	6	6	6	6	6	6	6
Mean	50.9	99.6	197.8	487.4	746.4	977.8	1959.4	4912.5	9552.8
±SD	2.5	4.6	6.8	12.9	23.2	18.5	51.8	242.1	371.6
Precision (% RSD)	4.9	4.6	3.4	2.6	3.1	1.9	2.6	4.9	3.9
Accuracy (%)	101.8	99.6	98.9	97.4	99.5	97.8	97.9	98.2	95.5

Table 3
Stability of Clanfenur (1.0 mg l^{-1}) in human plasma *in vitro* at 37°C

Time (h)	Mean observed concentration (ng ml^{-1})	% RSD	% of zero-time	Samples (*n*)
0	1020	0.6	100.0	6
0.5	1039	2.0	101.8	3
1.0	1026	1.4	100.6	3
2.0	1032	4.0	101.2	3
4.0	1028	2.0	100.8	3
17.0	986	1.0	96.7	2
24.0	883	4.5	86.6	4
48.0	776	3.5	76.1	3
72.0	560	7.6	54.9	3

much less work and time to obtain versatile information, using only 0.1 ml plasma samples. This procedure will be applied in monitoring the time course of absorption and elimination of the compound, to define the pharmacokinetics and metabolism both in the preclinical evaluation and during phase I trials.

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