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On-line continuous-flow dialysis thermospray tandem mass spectrometry for quantitative screening of drugs in plasma: rogletimide

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ABSTRACT

The application of a continuous-flow dialysis system, consisting of a membrane dialyser and a trace enrichment column, in on-line combination with tandem mass spectrometry via a thermospray interface is described. The method is applied to the quantitation of drugs in complex biological matrices containing macromolecular interferences. The potential of the method is demonstrated by the quantitative analysis of the anti-cancer drug rogletimide in the plasma of patients after treatment.

INTRODUCTION

The quantitation of drugs in biological fluids is an important aspect of drug development and testing, *e.g.* with respect to pharmacokinetics. In many cases, mass spectrometry (MS) is involved in these studies, often in combination with separation methods such as gas or liquid chromatography (GC or LC). The major reasons for using MS are related to its sensitivity and the ability to work with stable isotopically labelled internal standards. The use of tandem mass spectrometry (MS–MS), especially triple quadrupole instruments, in this field has been advocated by Yost and co-workers [1,2]. Effective screening of drugs and metabolites by the use of soft ionization techniques and highly selective MS–MS methods can be achieved in this way [1–3]. Similar approaches were described for environmental analysis by Hunt *et al.* [4]. One of the features of these approaches is that the biological samples are analysed without significant sample pretreatment, and at any rate without chromatographic separation. However, we feel that in the routine analysis of complex biological samples some sample pretreatment is obligatory prior to MS–MS in order to avoid source contamination. Great progress has been made in the development of the on-line coupling of liquid-phase methods to MS and MS–MS via interfaces for LC–MS. In this way it is possible to perform the necessary sample pretreatment on-line.

Continous-flow membrane dialysis (CFD), *i.e.* a combination of a membrane dialyser and a trace enrichment column [5–7], has been demonstrated to be an effective sample pretreatment method in the analysis of complex samples containing macromolecular interferences, *e.g.* plasma, especially when used in combination with a selective detection method. The plasma sample is introduced in a (buffered) aqueous donor stream, which is dialysed to

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an acceptor stream. The latter is subsequently enriched onto a short trapping column. The trapping column is eluted with an appropriate solvent and the eluate is directed to an analytical LC column for separation and subsequent detection. However, in combination with a highly selective detector such as a (tandem) mass spectrometer, the trapping column can be coupled directly via an LC–MS interface, without the need for an additional analytical separation, as has been demonstrated by our group in the phase system-switching approach [8,9].

In this paper the on-line combination of CFD and MS–MS via a thermospray interface is described. The potential of the approach is demonstrated by the quantitative bioanalysis of rogletimide in plasma.

Rogletimide, 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione (pyridogluthetimide, PG, **1** in Fig. 1), an analogue of aminogluthetimide, is under investigation as an aromatase inhibitor to be used in the treatment of post-menopausal women suffering from oestrogen-dependent breast carcinoma [10–12]. The human metabolism of PG has recently been elucidated with the use of thermospray LC–MS [13]. Some preliminary results regarding the detection of PG metabolites using the CFD–MS approach are reported here as well.

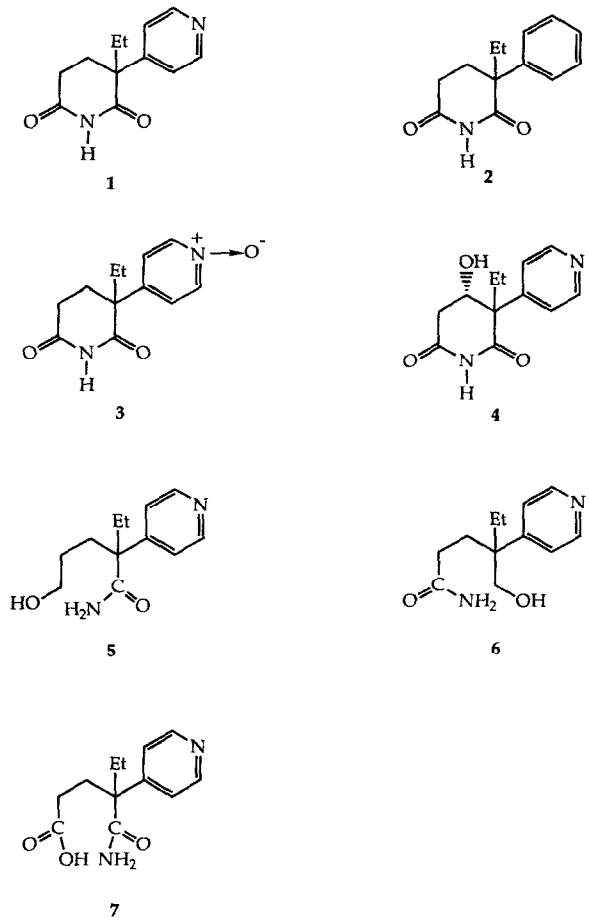

Fig. 1. Structures of the compounds investigated. **1** = Rogletimide (PG); **2** = gluthetimide; **3** = PG-N-oxide; **4** = 4-hydroxy-PG; **5** = 2-ethyl-2(4-pyridyl)-5-hydroxypentanamide; **6** = 4-ethyl-4-(4-pyridyl)-5-hydroxypentanamide; **7** = 2-ethyl-2-(4-pyridyl)-5-carboxypentanamide. Et = ethyl.

EXPERIMENTAL

Continuous-flow dialysis

The CFD system (see Fig. 2) consisted of a Skalar Analytical (Breda, Netherlands) Model 1000 autosampler, containing a Model 2002 multichannel peristaltic pump and a Model 5275 70-cm perspex dialysis block containing a cuprophan membrane (cut-off value 10 kilodaltons) coupled to two Rheodyne (Cotati, CA, USA) Model 7010 switching valves controlled by a Model 75A matrix timer (Kipp & Zonen, Delft, Netherlands). The laboratory-made trapping column (12 mm × 2 mm I.D.) was hand-packed under reduced pressure with 40–63 μm Polygosil C_8 particles (Machery-Nagel, Düren, Germany).

Plasma (1.26 ml) was introduced in an air-segmented (0.23 ml/min) donor stream (demineralized water, 0.42 ml/min) to the dialysis membrane. The non-segmented acceptor stream (demineralized water, 0.60 ml/min) was concentrated on the trapping column, which was subsequently desorbed for either liquid chromatography with UV detection, MS–MS with a thermospray interface, or LC–MS with a thermospray interface.

CFD in combination with LC and UV detection

The method was first developed and tested using a CFD system in combination with LC with UV detection. The eluate of the trapping column was directly introduced onto the analytical column. In these experiments, the LC system consisted of a Jasco (Tokyo, Japan) Model Familic 300S pump, a

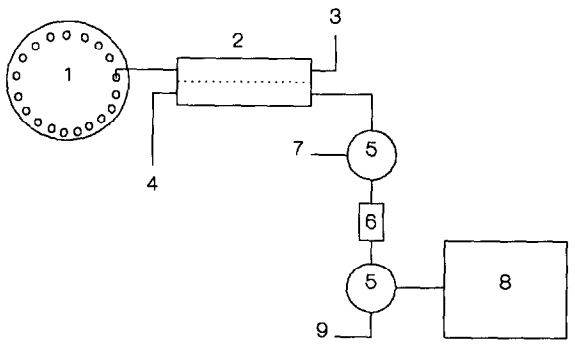

Fig. 2. Schematic diagram of the continuous-flow dialysis system coupled to tandem mass spectrometry via a thermospray interface. 1 = Autosampler; 2 = dialysis block; 3 = donor stream to waste; 4 = acceptor stream; 5 = switching valve; 6 = trapping column; 7 = desorption solvent; 8 = tandem mass spectrometer with thermospray interface; 9 = to waste.

Rheodyne Model 7125 injection valve equipped with a 20-μl injection loop, a Pye Unicam (Cambridge, UK) Model LC3 variable-wavelength UV detector operating at 254 nm and a Model BD40 flatbed recorder (Kipp & Zonen). The column was a 100 mm × 4.6 mm I.D. column slurry packed in the laboratory with 5-μm Nucleosil C_8 particles (Machery-Nagel). Isocratic elution was performed with a mobile phase of 30% methanol in 100 mmol/l ammonium acetate.

CFD in combination with a thermospray interface for LC–MS and MS–MS

The CFD system was coupled to a Finnigan MAT (San Jose, CA, USA) TSQ 70 tandem mass spectrometer equipped with a Finnigan MAT thermospray interface. The thermospray system was run with a mobile phase of 50% methanol in 50 mmol/l ammonium acetate at a flow-rate of 1.2 ml/min in discharge-on mode (1 kV). The vaporizer temperature and the repeller potential were optimized daily with respect to signal intensity and stability [14]; typical values were 100°C and 50 V, respectively. The ion source block temperature was kept at 200°C.

In MS–MS experiments, air was used as the collision gas (0.05 Pa). The collision energy was optimized at 60 eV. The effluent of the trapping column was introduced directly to the thermospray interface, bypassing the analytical column. In LC–MS experiments, the effluent of the trapping column was introduced into the analytical column, which was connected to the thermospray interface. The column and mobile phase were the same as used in the UV experiments.

Chemicals and sample treatment

Demineralized water was used throughout this study. Methanol and ammonium acetate were purchased from Baker (Deventer, Netherlands).

System development was performed using pooled plasma samples. Patients' plasma samples were collected before and 0, 0.5, 1, 2, 4, 6, 8, 12, 15, 24, 28, 32, 36 and 48 h after a single oral dose of 800 mg of PG. The samples were kept at −20°C for a prolonged period of time. Before use, they were defrosted at room temperature, diluted with demineralized water, and 10 μg of gluethetimide (Glu, **2** in Fig. 1) in 100 μl of methanol were added to 2 ml of plasma as an internal standard. Then, 1.26 ml of the sample were introduced via the autosampler without any further treatment. In quantitative studies, the plasma samples collected 0–12 h after administration were diluted twenty-fold and those collected 15–48 h after administration four-fold. In the preliminary studies with the metabolites no dilution was applied and no internal standard was added.

RESULTS AND DISCUSSION

Setting up the CFD system

In developing a CFD system for a particular application, several aspects must be taken in consideration, *e.g.* the selection of an appropriate column packing material for the enrichment of the dialysate and of a mobile phase. The latter should be capable of an efficient desorption of the compound of interest from the trapping column and should be favourable to the LC–MS interface. Furthermore, attention must be paid to the flow direction, *i.e.*, concurrent or counter-current dialysis and forward-flush or back-flush desorption from the trapping column, and to the optimization of the experimental parameters. The general importance of these parameters has been discussed by others [5–7,15,16]. In the present study, limited attention was paid to these aspects. No significant differences were observed between the various flow directions indicated above. Counter-current dialysis and forward-flush

desorption were used throughout the study. Efficient desorption of PG from the trapping column is possible with an aqueous mobile phase containing more than 20% methanol; generally 50% methanol was used. The presence of ammonium acetate had no influence on the desorption characteristics. The breakthrough volume of the trapping column for PG was found to be 10.2 ml, at a flow-rate of 0.5 ml/min, of 50 μg/ml PG in water, corresponding to a loading capacity of the trapping column for PG of *ca.* 500 μg, which is sufficient for the application at hand. PG shows poor UV characteristics; the on-column minimum detectable concentration in the CFD–LC–UV system is *ca.* 0.5 μg/ml (254 nm). Recovery after dialysis was found to be *ca.* 33%. The coefficient of variation in peak area on the UV detector was found to be 3.8% ($n = 5$) at the 10 μg/ml level.

The analysis time is *ca.* 14 min per sample. Although not actually performed, the CFD–MS–MS system can be run automatically and unattended when sufficient precautions in terms of control feedback are taken (see, for example, ref. 17).

Thermospray MS of PG

The mass spectrum of PG under thermospray conditions is characterized by an intense peak of the protonated molecule at *m/z* 219; some minor fragment peaks are observed as well. The detection limit of PG in column bypass injection without the CFD system is 50 ng/ml; good linearity is observed in the range 50 ng/ml to 20 μg/ml.

The product-ion spectrum of the protonated molecule of PG under thermospray MS–MS conditions is given in Fig. 3a. The base peak of the spectrum, *m/z* 134, most likely results from a cleavage of the piperidine ring with charge retention of the pyridyl ring ($C_9H_{12}N^+$). In the CFD–MS–MS analysis of standard solutions of PG, using selected monitoring of the reaction 219 → 134, good linearity was observed over the range 5–1000 ng/ml. The detection limit was *ca.* 4 ng/ml. This detection limit indicates that despite the dilution and the poor recovery in the dialyser a significant gain in concentration detection limit, relative to column bypass injections, is achieved as a result of the trace enrichment column.

Selection of an internal standard

Although good linearity was observed for PG under thermospray conditions, for a proper quantitation of PG in patients' plasma samples and internal standard should be used. As no isotopically labelled PG was available, an analogue of PG, *i.e.* gluthetimide (Glu, **2** in Fig. 1), was selected as internal standard. The thermospray mass spectrum of Glu is characterized by an intense peak at *m/z* 218 owing to the protonated molecule; no significant fragmentation is observed. Quantitatively, and somewhat surprisingly, the responses of Glu and PG under thermospray conditions are similar. The product-ion spectrum of the protonated molecule of Glu is given in Fig. 3b. The compound shows considerable fragmentation. The peak at *m/z* 131, which will be used in selected reaction monitoring, is most likely due to a piperidine ring cleavage, leading to an ion with an elemental composition of $C_9H_7O^+$.

Glu can also be used in the CFD system under the same conditions as used for PG. The loading

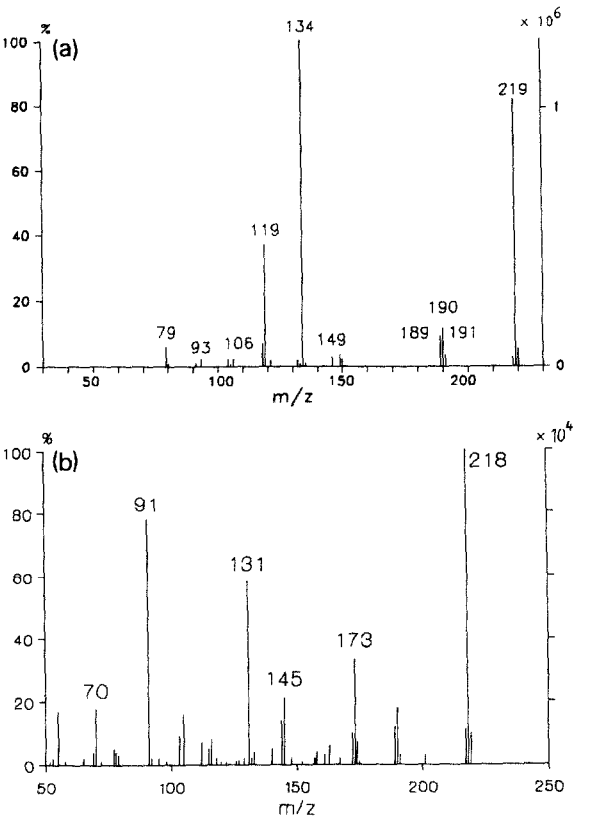

Fig. 3. Product-ion spectra of (a) rogletimide and (b) gluthetimide obtained after thermospray introduction; 200 ng injected. Right-hand scale: arbitrary units.

capacity of the trapping column for Glu was found to be *ca.* 350 μg, which is sufficient for this application.

Quantitative analysis of PG in plasma

After establishing the applicability of the method with spiked pooled plasma samples, the patient's plasma samples were analysed during the CFD–MS–MS approach in column bypass mode. Calibration of the method was done with spiked samples using the patients' plasma taken before the administration of PG. Calibration and patients' samples were measured in one series of experiments and in random order. The samples were analysed in selected reaction monitoring (219 → 134 for PG and 218 → 131 for the internal standard Glu). With spiked samples containing 1 μg/ml Glu and/or 0.8 μg/ml PG the possibility of mutual interferences in the selected reaction monitoring was experimentally excluded. Although a series of calibration samples could succesfully be measured without the addition of an internal standard, quantitation with an internal standard is preferred for the analysis of the post-treatment samples in order to correct for irregular ionization behaviour and other fluctuations in time.

With the series of calibration samples analysed with internal standard, good linearity was observed over the range 5–360 ng/ml. The determination limit of PG in plasma is found to be 5 ng/ml, indicating the effectiveness of the CFD as sample pretreatment in the analysis of real samples. The coefficient of variation in the peak area was found to be 5.2% ($n = 5$) at the 100 ng/mg level. In the post-treatment patients' samples, plasma concentrations of PG between 330 ng/ml and 40 μg/ml were observed. Therefore, dilution of the plasma samples was necessary to enable the measurement of the levels within the range of the calibration plot. A typical selected product-ion monitoring trace for a patient's plasma sample taken 36 h after administration is given in Fig. 4. The maximum plasma level found is *ca.* two-fold higher than that previously reported for PG [12] (see below).

A prerequisite of the applicability of the CFD approach is that the protein binding of the drug of interest is weak, *i.e.* readily reduced under conditions amenable to the CFD system. The plasma protein binding of PG was reported to be *ca.* 17%

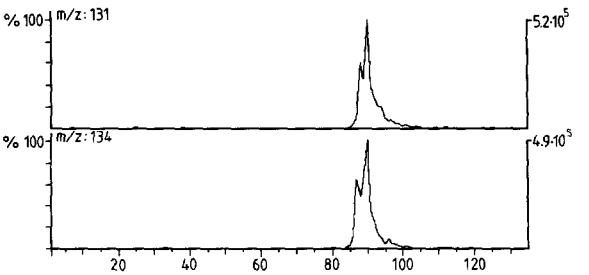

Fig. 4. Selected product-ion monitoring of the reactions for PG (*m/z* 219 → 1314) and Glu (*m/z* 218 → 131, internal standard) in CFD–MS–MS. Sample: four-fold diluted patient's plasma sample, 36 h after administration. Other conditions: see text.

and was not concentration-dependent [12]. Moreover, dilution of the plasma sample often reduces the protein binding. These effects were not further investigated for PG in this study.

Analysis of metabolites with the CFD system

In principle, the CFD approach can also be used to detect and quantitate drug metabolites in plasma. PG-N-oxide (**3** in Fig. 1) has previously been reported to be the principal metabolite of PG [11,12]. Plasma levels of PG-N-oxide up to *ca.* 2 μg/ml following a single oral dose administration of 1 g of PG have been reported [12]. The metabolism of PG was studied more extensively by Poon *et al.* [13] using thermospray LC–MS. Several metabolites were found in plasma, although some of them appeared to be present at low levels (assuming about equal response in thermospray ionization for the various metabolites). The most abundant metabolite was PG-N-oxide (**3**, molecular weight 234). Furthermore, 4-hydroxy-PG (**4**, molecular weight 234), 2-ethyl-2- and 4-ethyl-4-(4-pyridyl)-5-hydroxypentanamide (**5** and **6**, respectively, molecular weight 222) and 2-ethyl-2-(4-pyridyl)-5-carboxypentanamide (**7**, molecular weight 237) were found with significant abundance [13]. However, the last-mentioned compound is too polar to be trapped on the trapping column used in the CFD experiment.

The development of a CFD–MS system for the detection and quantitation of the metabolites of PG in plasma is hampered by the fact that several metabolites have the same molecular mass. The parent-ion spectra of the various metabolites in MS–MS also showed insufficient specificity for isomeric

discrimination by multiple reaction monitoring. Therefore, a chromatographic separation prior to the MS detection is needed. Some preliminary experiments with CFD coupled to an LC–MS system enabled the detection of two of the major metabolites, *i.e.* PG-N-oxide (**3**) and 4-hydroxy-PG (**4**). More extensive experiments, *e.g.* directed at investigating the loadability of the trapping column for these more polar metabolites and at quantitative aspects, are needed for a full assessment of the potential of the CFD approach in the quantitation of PG metabolites.

The work on the metabolites of PG revealed a possible source of overestimation of the PG level in plasma. The major metabolite, PG-N-oxide, is under thermospray conditions readily converted to PG; the intensity ratio of the peaks at *m/z* 219 and *m/z* 235 appears to be very sensitive to the conditions and not very reproducible. From this it may be concluded that part of the PG concentration measured in patients' plasma must be attributed to PG-N-oxide. In principle, this problem can obviated by the use of a short separation column between the CFD and the MS–MS, as PG and PG-N-oxide have significantly different retention characteristics. It must be emphasized that this unfavourable coincidence is typical for PG, which was chosen as the model compound for this study, but must not be considered detrimental for the CFD–MS–MS approach as such.

CONCLUSION

The method described here differs both in the experimental mode and in the field of application from the microdialysis systems which are used in the *in vivo* monitoring of, for instance, neurotransmitters [18]. Such systems have also been coupled to a mass spectrometer, either via a valve containing a loop to collect the dialysate [19] or directly to a low-flow LC–MS interface [20].

The CFD approach in on-line combination with MS and MS-MS via an LC-MS interface is an easy, rapid and versatile method for the quantitative monitoring of drug levels in biological samples. The method is useful if the presence of macromolecular compounds in the sample prohibits the direct introduction via a thermospray interface. No further sample pretreatment is necessary. The determination limits that can be achieved are compound-dependent, as is common with thermospray ionization. The method provides on-line sample preconcentration. An automatic sequential trace enrichment of dialysates system (ASTED) is commercially available from Gilson Medical Electronics (Villiers-le Bel, France).

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