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Optimization of a peak compression system for a remoxipride metabolite (FLA797) and its application to bioanalysis

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ABSTRACT

A peak compression system is optimized for FLA797 (**I**), a phenolic tertiary amine and a metabolite to the antipsychotic drug remoxipride. An application is described where this effect is used to give a 6–7-fold improvement of the quantification limit in an assay of **I** in plasma. The liquid chromatographic system is constructed so that the injection of **I** dissolved in a solution of a competing amine gives a very high and narrow analyte peak with an apparent efficiency of $1.5 \cdot 10^6$ plates/m. When the levels of **I** in plasma are determined, an internal standard, giving a normal isocratic peak, is added to the plasma sample before the extraction. Concentrations of **I** down to 0.5–1.0 n*M* can be determined with reasonable precision. FLA908, another phenolic remoxipride metabolite and a regioisomer to **I**, eluting as a normal isocratic peak, can be determined simultaneously although only at concentrations higher than 10–15 n*M*.

INTRODUCTION

Peak compression was introduced some years ago as a possible way to improve the quantification limits in liquid chromatography [1]. Amines were used as analytes. The columns were packed with octadecylsilica stationary phases with residual silanols, giving the addition of a competing amine to the mobile phase a significant influence on the retention of the amine analyte. The compressed peak was a consequence of the co-elution of the analyte peak with a system peak originating from the UV-transparent amine modifier. The UV-absorbing amine analyte was injected dissolved in a solution of a lipophilic organic anion. This anion generated a system peak with an amine modifier deficit, *i.e.*, a zone where the amine analyte was more strongly retained compared with the bulk mobile phase. The resulting analyte peak was very narrow. The effects on other analytes not co-eluting with the zone were small. These principles were applied in an assay of FLA908 (**II**), a remoxipride metabolite, in urine [2]. At that time, however, the lipophilicity of the amine modifier and the variation of the concentration of organic anion in the injected solution were the only parameters known that could be used for the optimization of a peak compression system for a given analyte. Both approaches are useful but have their drawbacks. Varying the lipophilicity of the amine modifier gives system peaks with very different capacity factors. Peak compression is seen for amine analytes having about the same capacity factor as the amine modifier, but not for the amine analytes with a differing retention. By increasing the concentration of the anion in the injected solution, the amine modifier deficiency zone is made larger and more amine analytes will be affected by the zone. This gives possibilities of obtaining compressed peaks in the same system for several amine analytes but, as they will all elute at the same time, at the expense of a decrease in selectivity.

It would be desirable to find means to change the capacity factor of the system peak relative to the capacity factor of the analyte. Such means were reported recently [3], resulting in an optimization strategy where the first step always is to find an

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amine modifier with about the same retention as the analyte. Then an octadecylsilica column with a suitable amount of residual silanols is chosen. This can be achieved by mixing stationary phases with different amounts of silanols. The final fine regulation can be made by adjusting the ionic strength of the phosphate buffer in the mobile phase, as the capacity factor of the system peak was found to increase and the capacity factor of the amine analyte decreased with increasing ionic strength.

Effects on peak shape, both compression and deformation, in similar systems have been studied by Johansson and co-workers [4,5] and Fornstedt *et al.* [6,7]. Peak focusing effects in injection-generated ion-pair gradients have been reported by Šlais *et al.* [8] and recently peak compression of trace components in displacement chromatography was described [9,10].

Remoxipride is a new antipsychotic drug, acting as a selective dopamine-D_2 receptor antagonist [11,12]. Remoxipride levels in plasma and urine were determined by reversed-phase high-performance liquid chromatography (HPLC) [13]. The compound is metabolized to a great extent in both man and animals [14] and also the phenolic metabolites **I** and **II** have been shown to be active at the dopamine-D_2 receptor [15]. These metabolites are present in only minute amounts and highly sensitive methods for their determination in biological fluids are therefore of great interest. In this paper, the optimization of a peak compression system for **I** is described. By injecting the analyte in a solution of the amine modifier also present in the mobile phase, a zone with an excess of amine modifier was created which was able to compress the analyte peak. The optimized system was applied to the determination of low levels of **I** in plasma. A system such as this is as simple to use as a normal isocratic system but takes a longer time to develop. The benefit is the possibility of achieving 5–10-fold improvements of the limit of quantification (LOQ). Other advantages and disadvantages are discussed.

EXPERIMENTAL

Chemicals

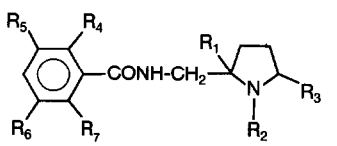
N,N-Dimethyldecylamine (DMDA) and N,N-dimethylnonylamine (DMNA) was obtained from Ames Labs. (Millford, CT, USA). DMDA was distilled before use. N,N-Dimethyloctylamine (DMOA) was obtained from Heraeus (Karlsruhe, Germany) and N,N-dimethyldodecylamine (DMDoA) from Aldrich-Chemie (Steinheim, Germany). Other chemicals, of HPLC or analytical-reagent grade, were obtained from the usual commercial sources and used as received.

Compounds **I**, **II** and **III** (the internal standard) were prepared at CNS Research and Development, Astra Arcus (Södertälje, Sweden). Their syntheses have been described [15]. Also some other similar compounds from the same laboratory (raclopride, remoxipride, **IV** and **V**) were used as model compounds. The structures are shown in Fig. 1. Standard solutions of these compounds were made in phosphate buffer (pH 2). The solutions of the phenolic compounds are slightly light sensitive and were therefore kept in the dark and prepared freshly every third week.

Chromatography

The columns (100 × 4.6 mm I.D.) were packed with 5-μm Spherisorb ODS-1 (Phase Separations, Queensferry, UK) from different batches. The packing was performed at 400 bar with methyl isobutyl phases as slurry medium and hexane as the eluent.

The mobile phases were mixtures of acetonitrile and phosphate buffer (pH 2) of different ionic strengths and with the addition of amine modifiers such as DMDA. The injected samples were dissolved in either neat phosphate buffer (pH 2) or in a solution of amine modifier in phosphate buffer.



	R_1	R_2	R_3	R_4	R_5	R_6	R_7
I	H	C_2H_5	2H	OCH_3	H	Br	OH
II	H	C_2H_5	2H	OH	H	Br	OCH_3
III	H	C_2H_5	2H	OCH_3	H	H	OH
Remoxipride	H	C_2H_5	2H	OCH_3	H	Br	OCH_3
Raclopride	H	C_2H_5	2H	OCH_3	Cl	Cl	OH
IV	H	H	2H	OCH_3	H	Br	OCH_3
V	OH	C_2H_5	O	OCH_3	H	Br	OCH_3

Fig. 1. Structures of the remoxipride-related compounds.

The chromatographic system consisted of a Waters Assoc. (Milford, MA, USA) Model 590 pump, a Perkin-Elmer (Überlingen, Germany) ISS-100 autosampler, with an injection volume always of 200 μl, and a Waters Model 470 fluorescence detector with a 5- or 16-μl cell volume. The excitation wavelength was 316 nm, slit width 18 nm, and the emission wavelength was 465 nm, slit width 30 nm. The shortest available time constant, 0.5 s, was used. A Perkin-Elmer LC-95 UV detector was also used. This detector was operated at 212 nm, the cell volume was 1.4 μl and the time constant was 0.02 s. The system peaks were followed with a Waters Model 410 refractive index (RI) detector coupled in series with the UV detector. The fluorescence and UV signals were monitored with an SP 4270 integrator (Spectra-Physics, San Jose, CA, USA) using the shortest available PW (peak width) value. The RI detector signal was monitored with a standard recorder. To prolong the lifetime of the analytical column, a membrane pulse damper (SP-21; Scientific Systems, State College, PA, USA) and a guard column (100 × 4.6 mm I.D.) dry-packed with Corasil (37–50 μm) (Waters Assoc.) were placed between the pump and the autosampler.

The apparent efficiencies of the compressed peaks were calculated using the peak width at half-height. Peak asymmetry factors were calculated at 10% of the peak height.

*Determination of **I** and **II** in plasma*

Plasma samples (0.50–1.00 ml) were adjusted to pH 9–10 by the addition of an equal amount of 0.1 *M* carbonate buffer (pH 10). The appropriate amount (50–100 pmol) of internal standard (**III**) and 4 ml of 20% *n*-hexane in diethyl ether were added. The sample tubes were extracted in a rotary mixer for 5 min and then centrifuged for 2 × 10 min at 1500 *g* and 2°C. The organic phase was transferred into another tube and evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 250 μl of 0.8 m*M* DMDA in 0.039 *M* phosphate buffer (pH 2) ($I = 0.015$) and 200 μl were injected.

The column was packed with Spherisorb ODS-1, batch 29/81. The mobile phase was 31% acetonitrile in 0.039 *M* phosphate buffer (pH 2) ($I = 0.015$) with the addition of 0.5 m*M* DMDA. Fluorescence detection was used.

The unknown concentrations of **I** and **II** were calculated from a linear calibration graph consisting of 6–8 spiked plasma samples. To study the absolute recoveries, extractions were performed using aliquots. The absolute recovery was calculated by dividing the slope of the concentration *versus* peak height graph by the slope of a calibration graph obtained from direct injection of aqueous standard solutions of the compounds. The precision and accuracy were studied by spiking a number of blank plasma samples with known amounts of **I** and **II**. Thereafter the samples were analysed as described above.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

When a peak compression system is to be optimized for a specific analyte, the first step is the choice of an amine modifier with about the same retention as the analyte, *i.e.*, giving a capacity factor ratio as close to unity as possible. The second step is the choice of a column with the appropriate amount of residual silanols and the third step is to adjust the ionic strength of the phosphate buffer of the mobile phase to give a capacity factor ratio of 1.00 [3]. Other parameters, such as acetonitrile concentration, amine modifier concentration and pH, were found to have a minor or negligible influence on the capacity factor ratio for this kind of system [3].

When DMNA, DMDA and DMDoA were used as amine modifiers in otherwise similar mobile phases, their system peaks gave capacity factor ratios, relative to **I**, of 0.48, 1.04 and 4.38, respectively. DMDA was therefore the most appropriate amine modifier for **I** and it was used in a mobile phase concentration of 0.5 m*M*. A concentration of 31% acetonitrile in the mobile phase gave a practical retention time.

Then, ideally two octadecylsilica (ODS) materials with a large difference in the residual silanol content were mixed to give a suitable column. As a measure of the silanol content the ratio of the capacity factor for the system peak to the capacity factor for an amine analyte (remoxipride) was used. Earlier [3], Spherisorb ODS-1, having a ratio of 1.93, and ODS-2, with a ratio of 4.36, were used. As small variations in the ionic strength of the mobile phase might have a drastic effect on the capacity factor

ratio at low ionic strengths, it was proposed earlier that peak compression effects at high ionic strengths would possibly be more robust [3]. Preliminary tests with I showed that the ideal ODS-1 material should have a capacity factor ratio of about 2 in the experiment above, then a high ionic strength could be used. Unfortunately, the currently available ODS-1 batches gave higher ratios, between 2.6 and 3.4. Therefore, the ODS-1 batch with the lowest ratio (batch 29/81) was chosen, but had to be used unmixed and combined with a low ionic strength of the mobile phase buffer. As a consequence, part of the flexibility in the design of the peak compression system was lost.

The third step in the optimization strategy was to find a situation where the capacity factor of the system peak exactly equals the capacity factor of the analyte, and this is done by adjusting the ionic strength of the mobile phase buffer. A capacity factor ratio of unity was found at $I = 0.015$–0.020. No negative effects of using this low ionic strength were observed.

The results so far were obtained by the injection of I in neat buffer. By using this technique a small positive DMDA system peak, the height depending on the DMDA concentration in the mobile phase, was generated in agreement with, *e.g.*, ref. 16. Also, this small system peak gave a compressed peak for I, but with good apparent efficiency ($N_{app}/m > 10^6$) only when very small amounts were injected (linearity is discussed further below). A larger compressing zone can be generated in several ways, two of them being the injection of an anion more lipophilic than DMDA or by injection of DMDA itself. The first approach, used in earlier work [1,2,4–7], gives a zone with a DMDA deficit and with a higher capacity factor than the small positive peak. This negative peak can give efficient compression but often also split peaks and other deformations. Another disadvantage is that the peak containing the lipophilic anion must be eluted before the next injection is made. Hence the sample throughput becomes lower than with an isocratic system. The injection of DMDA supplements the DMDA excess generated by the injection of buffer and this larger excess also has a shorter retention then the system peak from a buffer injection. The retention depends on the DMDA concentration in the peak as the adsorption isotherm for DMDA is non-linear under the conditions described. The positive DMDA peak was found to be the best way to generate peak compression effects as it gives efficient compression and also gives a fast system without late-eluting anion peaks.

The optimization described so far has been focused on giving I and the system peak the same capacity factor, the prerequisite for peak compression effects. To optimize also the efficiency of the compressed peak, the influence of the amine modifier concentration in the injected solution must be studied. In Fig. 2 the influence of the DMDA concentration on the apparent efficiency for I is shown. Under these conditions an optimum was found at 1.5 m*M* DMDA, where the apparent efficiency (N_{app}/m) was about $1.75 \cdot 10^6$ plates.

In conclusion, in the optimized system a column with unmixed Spherisorb ODS-1, batch 29/81, was used together with a mobile phase consisting of 31% acetonitrile in phosphate buffer (pH 2, ionic strength 0.015) containing 0.5 m*M* DMDA. The sample was dissolved in a solution of 1.5 m*M* DMDA in phosphate buffer. The resulting chromatographic peaks for I and II are shown in Fig. 3. A high chart speed was used to demonstrate the peak shape.

Optimization of detectability

The width of a compressed peak in the discussed

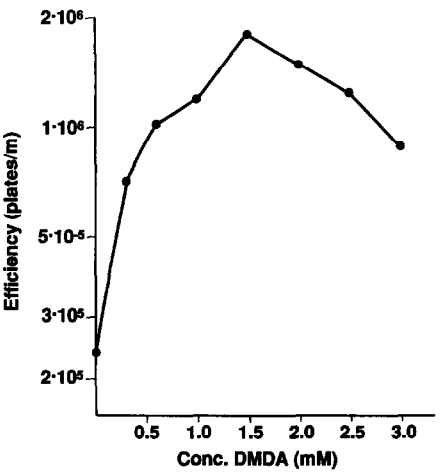


Fig. 2. Influence of DMDA concentration in the injected solution on the chromatographic efficiency for I. Mobile phase: 31% acetonitrile in phosphate buffer (pH 2, $I = 0.015$) containing 0.4 m*M* DMDA. Sample: I (1 μM) dissolved in 0–3 m*M* DMDA, 200 μl injected. UV detection. Flow-rate, 1.0 ml/min.

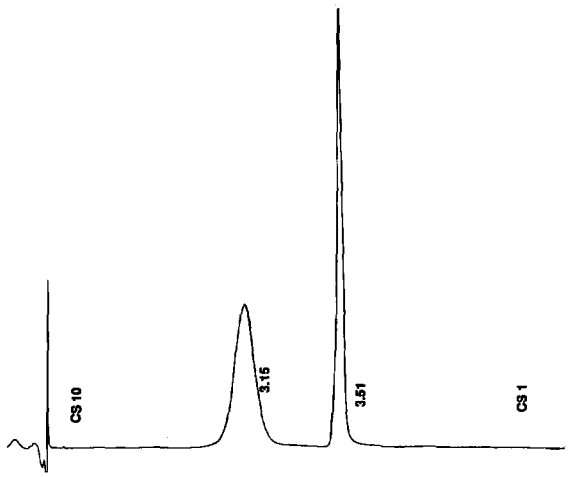


Fig. 3. Peak shapes for the isocratic compound **II** peak and for the compressed compound **I** peak. Sample: **II** (2 μM) and **I** (1 μM) dissolved in 1.5 mM DMDA. Other conditions as in Fig. 2. Programmed chart speed (CS): 0–2.5 min, 10 mm/min; 2.5–4.0 min, 100 mm/min; 4.0–6.0 min, 10 mm/min. Numbers at peaks indicate retention times in min.

systems is typically 10–20 μl and necessitates a detector with a small cell volume and a short time constant. In the optimization of the present system, UV detection with a detector cell volume of 1.4 μl and a time constant of 0.02 s was used, giving negligible loss in efficiency for most peaks. When attempts were made to use the peak compression system combined with UV detection at high sensitivity and for quantitative purposes, a large blank disturbance was seen when DMDA was injected. This peak had the same retention as **I** and the system peak. The peak height depended on the injected concentration of DMDA and was probably a result of a low UV absorbance of DMDA itself and/or an amine impurity. Distillation of DMDA did reduce, but not remove, the peak. Using the optimized system described above and a flow-rate of 1.0 ml/min, the peak height of the blank disturbance corresponded to an injected amount of 2 pmol of **I**. This blank disturbance was unacceptable.

The phenolic metabolites to remoxipride also have natural fluorescence, however, and the UV detector was replaced with a fluorescence detector with a standard 16-μl flow cell and the shortest time constant of 0.5 s. The blank disturbance was reduced, now corresponding to 200 fmol of **I** or about four times the noise level of the fluorescence signal. Obviously, this cell is much too large and the apparent efficiency for the **I** peak was reduced from $N_{app}/m = 1.75 \cdot 10^6$ (UV) to $0.64 \cdot 10^6$. At the same time, the peak asymmetry factor (Asf) increased from 1.5 to 2.4. Replacing the original cell with a 5-μl cell and connecting the column directly to the cell improved both the efficiency ($N_{app}/m = 0.8 \cdot 10^6$) and the symmetry ($Asf = 2.0$), but still about 50% of the efficiency was lost. As the asymmetry of the peak still was markedly higher than that of the peak from the UV detector, the flow characteristics of the cell were thought to be inadequate and the flow-rate was reduced to 0.5 ml/min, resulting in $N_{app}/m = 1.25 \cdot 10^6$ and $Asf = 1.8$. An interesting effect of the reduction in flow-rate was that the optimum efficiency was found at a lower amine modifier concentration, possibly owing to kinetic effects in the contributing equilibria. This is demonstrated in Fig. 4, where the apparent efficiency is plotted against the DMDA concentration in the injected solution. Using the fluorescence detector and a flow-rate of 0.5 ml/min, the optimum ($N_{app}/m = 1.45 \cdot 10^6$ with $Asf = 1.8$) was found at 0.8 mM DMDA. This can be compared with $N_{app}/m = 1.75 \cdot 10^6$ and $Asf = 1.5$ for the optimized UV system. The losses in efficiency and symmetry are due to the larger cell volume and the higher time constant of the fluorescence detector. The influence of cell

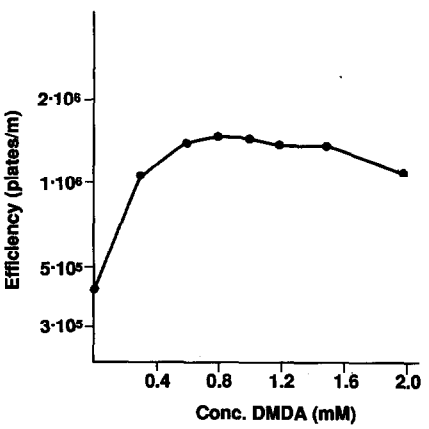


Fig. 4. Influence of DMDA concentration in the injected solution on the chromatographic efficiency for **I**. Mobile phase: 31% acetonitrile in phosphate buffer (pH 2, $I = 0.015$) containing 0.5 mM DMDA. Sample: **I** (1 μM) dissolved in 0–2 mM DMDA, 200 μl injected. Fluorescence detection. Flow-rate, 0.5 ml/min.

volume and time constant on the peak broadening can be calculated using the equations introduced by Martin *et al.* [17]. The 20% loss in efficiency found, corresponding to a peak broadening of about 10%, is considerably lower than the value calculated using these equations, partly owing to the overestimation of the plate number for asymmetric peaks. As the optimum efficiency was found at a lower injected concentration of DMDA, the blank disturbance was also lowered to the same extent, now corresponding to an injected amount of about 100 fmol of **I** or twice the noise level.

The total result of changing detection principle was a 20-fold reduction of the blank disturbance, but also a loss of about 20% in the number of theoretical plates and an increase asymmetry of the peak.

Selectivity in peak compression systems

An inherent disadvantage with peak compression systems is the loss of selectivity in the zone where the analyte peak is compressed. All injected amines, and probably also other compounds with an ability to interact with silanols, with capacity factors covered by the zone will elute in the same peak. Obviously, the zone should be kept as small as possible. The importance of keeping the zone small is demonstrated in Fig. 5, where the internal standard, **II**, **I** and the later eluting raclopride were injected dissolved in increasing concentrations of DMDA. The system peak co-eluted with **I** at all concentrations. Without DMDA in the injected solution all four peaks were well separated. At DMDA concentrations $\geqslant 2$ mM, **I** and raclopride eluted together in a compressed peak and at 4 mM also **II** was trapped in the DMDA zone. The chromatograms at 0.5 and 2.5 mM DMDA are shown in Fig. 6. The peak shape for the last-eluting compound at the low DMDA concentration can be noted. This analyte has about the same retention as the zone within the zone, but considerably slower retention in the bulk mobile phase. When the analyte molecules reach the mobile phase behind the zone they will start to move

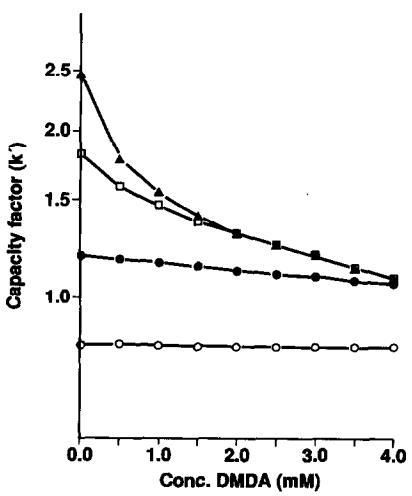


Fig. 5. Influence of DMDA concentration in the injected solution on the selectivity. Mobile phase, flow-rate and detection as in Fig. 4. Sample: (○) **III** (0.5 μM), (●) **II** (0.5 μM), (□) **I** (0.2 μM) and (▲) raclopride (0.5 μM) dissolved in 0–4 mM DMDA.

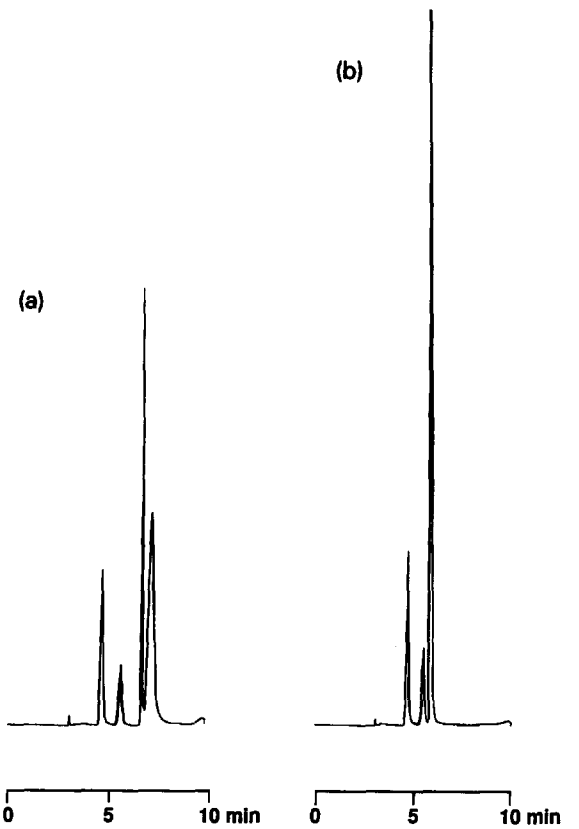


Fig. 6. Chromatograms demonstrating the change in selectivity with increase in the size of the compressing zone. Conditions and elution order as in Fig. 5. Sample dissolved in (a) 0.5 mM and (b) 2.5 mM DMDA.

more slowly than the zone and, consequently, peak broadening will occur.

Compounds with no ability to interact with silanols are not affected at all by the amine modifier zone, *i.e.*, neither the capacity factor nor peak shape is influenced. If the isocratic peak of such a compound elutes together with the compressed peak, the amine modifier concentration in the injected solution can be adjusted in order to make these peaks separate. This possibility of regulating the selectivity between amines and non-amines by varying the amine modifier concentration in the injected solution is demonstrated in a similar system where remoxipride and a lactam metabolite (**V**) eluted as one peak when dissolved in neat phosphate buffer (Fig. 7a). When the sample was dissolved in 2 m*M* DMOA the remoxipride peak instead was eluted as a compressed peak in front of the lactam (Fig. 7b). The retention of the lactam, and also that of the early eluting amine **IV**, were unchanged.

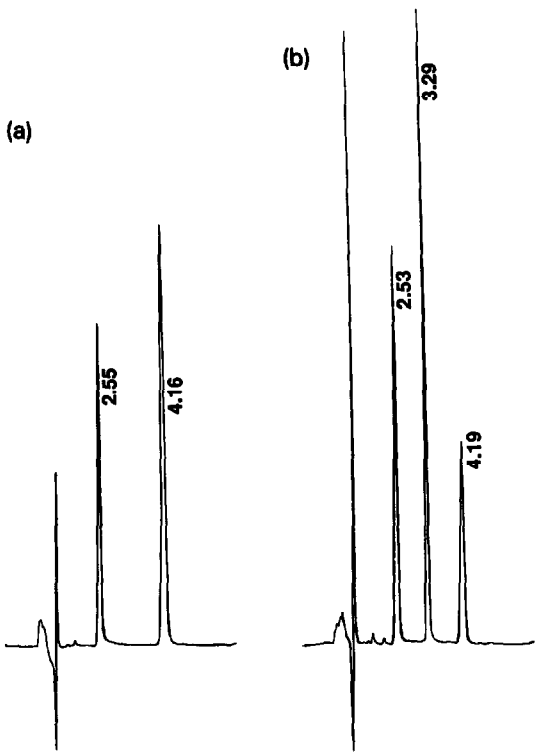


Fig. 7. Chromatograms showing the selectivity towards compounds not interacting with residual silanols. Column: Spherisorb ODS-1, batch 28/25. Mobile phase: 26% acetonitrile in phosphate buffer (pH 2, $I = 0.04$) containing 0.6 m*M* DMOA. Sample: **IV**, remoxipride and **V**, 1 μM each. (a) Sample dissolved in neat buffer; **IV** peak at 2.55 min and combined peak of remoxipride and **V** at 4.16 min. (b) Sample dissolved in 2 m*M* DMOA; **IV** peak at 2.53 min, remoxipride peak at 3.29 min and **V** peak at 4.19 min.

Compounds **I** and **II** are the two most lipophilic of the remoxipride metabolites and they are also considerably more retained than remoxipride itself. Therefore, when analysing samples from subjects treated with remoxipride, no remoxipride-related compounds will interfere with the **I** peak. The loss of selectivity in the present system was minimized as a concentration of only 0.8 m*M* DMDA was used to generate a compressed peak. Combining this with the selective fluorescence detection, the risk of false peaks for **I** must be considered to be very low.

Linearity

The linearity of the peak compression system was found to be limited and dependent on the concentration of the amine modifier in the injected solution. In Fig. 8, peak height for **I** is plotted against injected amount at three different amine modifier concentrations. When **I** was dissolved in 2 m*M* DMDA a straight line was obtained up to about 300 pmol of **I** injected. When dissolved in 1 and 0.3 m*M* DMDA, the plot was linear up to about 200 and 100 pmol, respectively. The non-linearity above these concen-

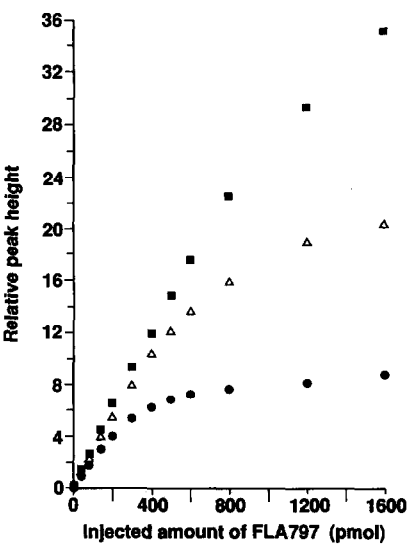


Fig. 8. Linearity in peak compression systems. Mobile phase as in Fig. 4, flow-rate 1.0 ml/min and UV detection. Sample: **I** (0–8 μM) dissolved in (●) 0.3, (△) 1.0 and (■) 2.0 m*M* DMDA.

trations was due to peak broadening. The mechanism behind the limited linear range is not understood but some additional experimental data can be given to describe the non-linearity. The height of the DMDA peak depends on both the injected amount and the DMDA concentration in the mobile phase. The linear range correlated well with the height of the DMDA peak, measured from the RI detector signal, which was 10.5 mm (0.3 m*M* DMDA), 19 mm (1.0 m*M* DMDA) and 31 mm (2.0 m*M* DMDA). The concentration at the peak maximum (C_{max}) can be calculated for both the system peak and the analyte peak using the equation

$$C_{max} = \frac{\sqrt{N_{app} m}}{\sqrt{2\pi} V_R} \quad (1)$$

where m is the injected amount and V_R the retention volume. If the found values for each parameter (N_{app} and V_R for the DMDA peak decreases with increasing injected amount of DMDA) were used, C_{max} in the DMDA peak was calculated to be 270, 490 and 790 μM, respectively, at the three injected DMDA concentrations. The corresponding peak maximum concentrations for the analyte peak were 3.9, 8.8 and 14.9 μM, when calculated on the highest concentration in the linear range. The results suggest that, for efficient peak compression to occur, the excess of amine modifier over the analyte must be >60. A lower ratio of the peak maximum concentrations results in peak broadening.

The linearity for the isocratic peak for **II** was also found to be limited, but this limitation was independent of the DMDA concentration. Injected amounts above 1200 pmol gave deviations from the straight line at all three DMDA concentrations. An experiment was also performed to establish whether high amounts of **II** influenced the linearity of **I**. No such influence could be seen.

It can be noted that if the sample is injected dissolved in a high concentration of the amine modifier, this will improve the linearity but decrease the selectivity and increase the blank disturbance. Consequently, the amine modifier concentration is always a compromise but normally the linearity will be sacrificed in favour of selectivity and sensitivity.

Optimization of the plasma extractions

Compounds **I** and **II** and also the internal standard (**III**) are both tertiary amines and phenols. The plasma extractions were performed at pH 9–10, where these groups are not ionized. Under these conditions, and using an organic phase of 20% *n*-hexane in diethyl ether, the absolute recovery was quantitative (>95%) for all three compounds. When the alkalinization was effected with sodium hydroxide, an addition to the blank disturbance described above was seen. This blank disturbance corresponded to an injected amount of 1 pmol of **I**. If the sodium hydroxide was replaced with carbonate buffer, the total blank disturbance was reduced to 200 fmol. These blank disturbances were equally large also when the aqueous phase in the extraction was water instead of plasma, indicating that plasma itself gave no contribution. As the blank disturbance was related to the amount of DMDA in the injected sample and the contribution from the carbonate buffer, two parameters that are easily controlled as they are the same for all samples in a set, the blank disturbance was found to be sufficiently reproducible not to hamper seriously the plasma determinations.

Repeatability and limit of quantification

To demonstrate the repeatability of the peak compression effect itself, an experiment was performed in which 32 consecutive injections of the same solution were made. This solution contained **I** (0.5 μM) and **II** (1 μM) dissolved in 1.5 m*M* DMDA. UV detection was used, the flow-rate was 1.0 ml/min and the heights for each peak were measured. When the relative standard deviations (R.S.D.) were calculated for the whole run, the R.S.D. for the peak height of the compressed peak was good (1.6%), although that for the isocratic peak was lower (0.7%). A trend for increasing peak heights during the run was observed for both compounds. This was probably due to evaporation of water as the autosampler vials were not capped. If the precision instead was calculated for ten consecutive injections, the mean R.S.D. was about 0.4% for the isocratic peak and 0.5% for the compressed peak. This appears to be as good as for normal isocratic systems when using the described autosampler.

The above results also showed, as both the compressed and the isocratic peak gave similar precision, that it would be possible to use another isocratic peak as internal standard for both com-

TABLE I

WITHIN-RUN PRECISION AND ACCURACY FOR PLASMA DETERMINATIONS OF I AND II

Spiked plasma samples were analysed as described under Experimental. The peak heights were measured by the integrator.

Compound	Accuracy		Precision, R.S.D. (%)	*n*
	Added concentration (n*M*)	Found concentration (n*M*)		
II	200	201	2.4	10
I	25.0	25.4	2.3	10
II	10.0	10.8	17	10
I	1.00	1.00	11	10
I[a]	1.00	1.08	5.2	10

[a] Manually measured peak heights.

pounds when determining their concentrations in plasma. In a peak compression system with an excess of amine modifier as compressing zone, an internal standard is preferably eluted before the compounds of interest because, as discussed above, amine peaks eluting closely after the system peak are broadened.

The phenolic compound **III**, similar to **I** and **II** but not a metabolite to remoxipride, had a suitable capacity factor and was chosen as the internal standard. The within-run precision and accuracy for spiked plasma samples, using the internal standard and extraction and analysis as described under Experimental, are presented in Table I. At the higher concentration the precision was about 2% for both compounds. At the low concentration, manual measurement of the peak heights for **I** gave a higher precision than using the peak heights reported by the integrator. For very small peaks it seemed easier to obtain a good measure of the distance between the peak apex and the average noise when they were measured manually. The results also showed that plasma extracts did not influence the chromatography. A calibration graph, ranging from 1 to 40 n*M* of **I**, is presented in Fig. 9, and Fig. 10 shows one of the chromatograms from that graph. A chromatogram for a real sample with a low concentration of **I**, 0.6 n*M*, and an unmeasurable concentration of **II** is shown in Fig. 11. In this chromatogram it can be noted that about one third of the peak height originated from the blank disturbance and also that

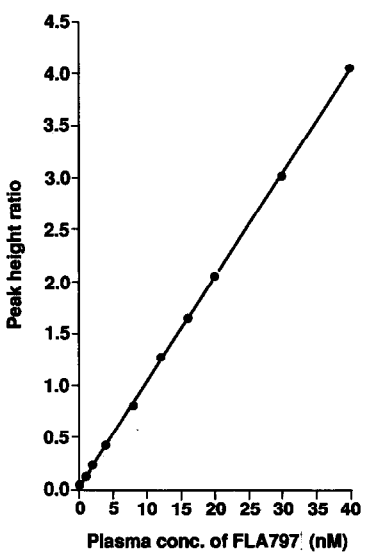


Fig. 9. Calibration graph for the determination of **I** in plasma. Sample work-up and chromatography as described under Experimental. Found intercept: −0.35 n*M*; correlation coefficient (R^2) = 0.9997.

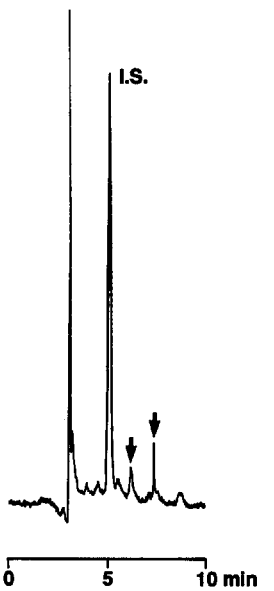


Fig. 10. Chromatogram of a standard sample. Sample work-up and chromatography as described under Experimental. Added concentrations: **II** = 5.0 n*M*, **I** = 1.0 n*M* and **III** (internal standard, I.S.) = 75 n*M*. The arrows indicate the positions of **II** (first arrow) and **I** (second arrow).

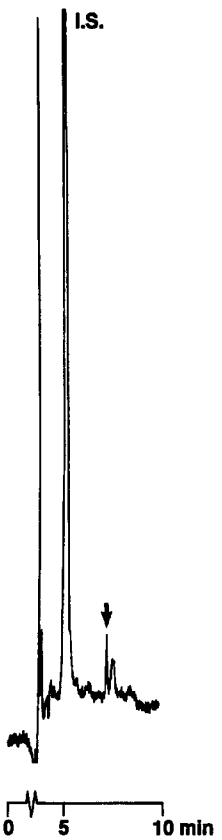


Fig. 11. Rat plasma chromatogram, sample taken 20 min after intraperitoneal administration of 40 μmol/kg of remoxipride. Sample work-up (plasma volume 635 μl) and chromatography as described under Experimental. The arrow indicates the position of **I**, found concentration 0.6 n*M*; internal standard (I.S.) concentration, 100 n*M*.

an isocratic endogenous peak eluted close to **I** without disturbing its peak height.

Based on the reported precision, it seemed possible to determine plasma concentrations of **I** down to 0.5–1.0 n*M* with reasonable precision while the LOQ for **II** was 10–15 n*M*. As **I** has a three times better fluorescence response than **II**, the peak compression effect can be estimated to give 6–7 times better sensitivity for **I** than an isocratic system with the same instrumental set-up.

Advantages and disadvantages of peak compression systems

Although more difficult to develop, this work shows that peak compression systems utilizing an excess of amine modifier as a compressing zone are equivalent to conventional isocratic systems in terms of reproducibility, sample capacity and simplicity. Using this kind of peak compression system, the only practical difference is that the sample is injected dissolved in a solution of the amine modifier and the only visible difference is that one peak is very narrow. Other compounds giving isocratic peaks can be determined simultaneously or added to the sample and used as an internal standard. The height of the compressed peak was shown to be as reliable and reproducible as the height of an isocratic peak, although the high efficiency of the compressed peak seemed to make it more sensitive to the condition of the column. This kind of system can also be compared with the displacement systems for peak compression [10]. In the displacement systems, the sample is injected into a column with a pure mobile phase and thereafter a mobile phase containing the displacer is introduced. The degree of compression seems to be about the same but the selectivity is low in the displacement system as a high concentration of a general displacer is used. On the other hand, the peak compression is not restricted to amines. The major drawback with the displacement systems is the need for regeneration of the column between each run, making these systems impractical for routine determinations of trace levels.

There are, however, two important limitations with the peak compression systems described in this paper: the requirement for a non-standard detector and the limited linearity. Although the linearity of a peak compression system is limited, the linear range for the described application is large enough (0.5–200 pmol) for the intended samples. If high concentrations (>200 n*M* of **I** in plasma) must be determined, the samples are simply diluted before injection or a normal isocratic system is used. In conventional HPLC, cell volumes of 8–15 μl and time constants of 1.0 s or more are used. The sharp peaks obtained with peak compression systems make it impossible to use these conditions. Although smaller cells are available, the optical system is usually not optimized for these cells, resulting in a larger decrease in signal-to-noise ratio than is expected from the difference in cell length/volume. Often also the stray light becomes higher when a smaller cell is used, leading to limitations in linearity. The peak compression effect improved the sensitivity for **I** by

6–7 times compared with an isocratic system with the same instruments. However, if the large 16-μl cell and a time constant of 1 s were used in the isocratic system, the signal-to-noise ratio would be improved, giving an LOQ almost comparable to that with the peak compression system. Until more optimum fluorescence detectors are commercially available, perhaps based on laser sources, the benefits of the described peak compression system regarding sensitivity enhancement cannot be fully utilized.

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