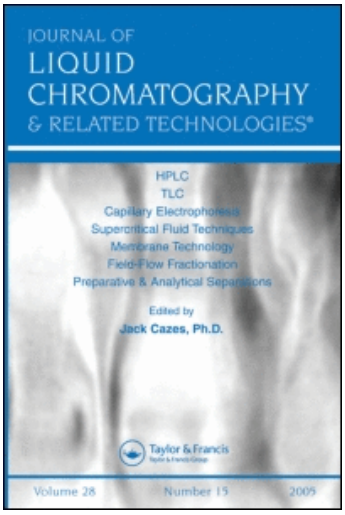

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LIQUID CHROMATOGRAPHIC DETERMINATION OF A SUBSTITUTED BENZAMIDE IN BIOLOGICAL FLUIDS USING PRECONCENTRATION AND POST-COLUMN EXTRACTION

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ABSTRACT

A sensitive and selective semi-automated method has been developed for the determination of Remoxipride in biological fluids. The chromatographic behaviour of the basic drug is strongly improved by treatment of the C-18 bonded column with hexadecyltrimethylammonium bromide. The use of disposable precolumns permits the direct injection of 1:1 (v/v) diluted plasma or 1:9 (v/v) diluted urine. For detection, a post-column ion-pair extraction with the strongly fluorescent 9,10-dimethoxyanthracene-2-sulphonate was used. A new sandwich type phase separator helped to keep band broadening very low. The calibration curves of Remoxipride in plasma and urine show correlation coefficients of 0.9999 over at least two orders of magnitude. Detection limits are 1 ng/ml in plasma and 15 ng/ml in urine. The recovery of Remoxipride from spiked plasma and urine is 88% and 76%, respectively. The reproducibility, based on peak height measurements, for determinations in plasma (200 ng/ml) was 3.5% (RSD) within one serial run (n = 5), and 3.5% (RSD) from day to day (n = 12). For determinations in urine (160 ng/0.2ml) the reproducibility within one serial run was good, i.e., 2.2% (RSD), whereas it was rather poor from day to day, i.e., 20% (RSD).

INTRODUCTION

Remoxipride ((S-(-)-3-bromo-N-[(1-ethyl-2-pyrrolidinyl)methyl]-2,6-dimethoxybenzamide hydrochloride monohydrate) (for structure, see Fig. 1) is a highly selective dopamine antagonist with neuroleptic properties [1]. In order to perform pharmacokinetic studies, a sensitive and selective method is required for the determination of this drug in biological fluids. Major problems in developing such a method are its UV absorbance which is high only at low wavelengths ($\varepsilon = 3.6 \times 10^4$ at 208 nm), where selectivity is poor, the absence of natural fluorescence and its poor chromatographic performance on silica-based packing materials in LC, which is often observed with solutes containing amine functional groups [2].

Post-column reaction detection for the enhancement of sensitivity and selectivity is becoming increasingly important in LC. One of the techniques used is based on ion-pair extraction of a solute with a strongly fluorescent counter-ion [3,4]. Compounds containing tertiary amino or quaternary ammonium groups easily form ion-pairs with 9,10-dimethoxyanthracene-2-sulphonate (DAS), which is strongly fluorescent [3,5,6]. By adding a non-miscible organic solvent after the reversed phase separation, a solvent-segmented system is formed which permits on-line extraction of the ion-pair and subsequent fluorescence detection. Unfortunately, the reversed-phase system developed by Nilsson [7] is not compatible with post-column DAS extraction detection since an amine modifier is used, which can also form ion-pairs with DAS and, thus, causes an extremely high fluorescence background. In the quoted paper, sample preparation consisted of a protein precipitation step followed by centrifugation, making internal standardization nescessary, as was also reported recently for the LC determination of another new benzamide in biological fluids [8].

In the present study we attempted to modify the method for the detemination of Remoxipride in biological fluids, by introducing a reversed-phase LC system, which is compatible with post-column DAS extraction detection. In addition, the use of precolumn technology, i.e., of disposable precolumns containing large-particle non-polar packing material was investigated which will permit the direct injection of pure or diluted biological fluids [6,9].

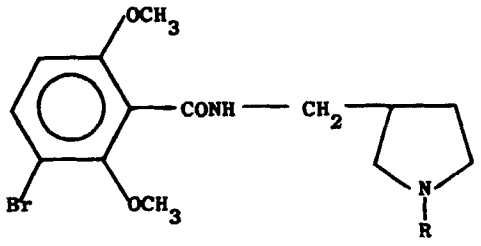
EXPERIMENTAL

Chemicals

Remoxipride and the internal standard FLA 913 (see Fig. 1) were received as a gift from Astra Alab AB (Södertälje, Sweden). HPLC-grade acetonitrile, analytical grade 1,2-dichloroethane (which can be recycled by distillation) and 1,4-dioxane were purchased from Baker (Deventer, The Netherlands). All aqueous solutions were prepared with demineralized water, treated in a Milli Q, (Millipore, Bedford, MD, USA) ultrafiltration system. 9,10-Dimethoxyanthracene-2-sulphonate sodium salt was obtained from Fluka (Buchs, Switzerland) and HPLC-grade hexadecyltrimethylammonium bromide (Cetrimide) from Fisions (Loughborough, Leics, UK). Fresh frozen human plasma was received as a gift from the Blood Transfusion Service of the OLVG Hospital (Amsterdam, The Netherlands).

Liquid Chromatography

The apparatus used is schematically shown in Fig. 2. The eluent and organic extractant were delivered by two Kontron (Zürich, Switzerland) LC 414 pumps with Kontron pulse dampers. In conjunction with the Kontron pulse damper, a 100 x 4.6 mm i.d. column, packed with a 10 µm C-18 bonded phase, was installed for optimal pulse damping of the organic extractant stream. The reagent was delivered by a Waters (Milford, MA, USA) Model 6000 A LC pump with a home-made membrane pulse damper. Preconcentration was done using either a Valco (Houston, TX, USA) six-port valve, equipped with a home-made 2 x 4.6 mm i.d.



	R
REMOXIPRIDE	C_2H_5
FLA 913 (I.S.)	C_3H_7

Figure 1.
Structures of Remoxipride and the internal standard FLA 913

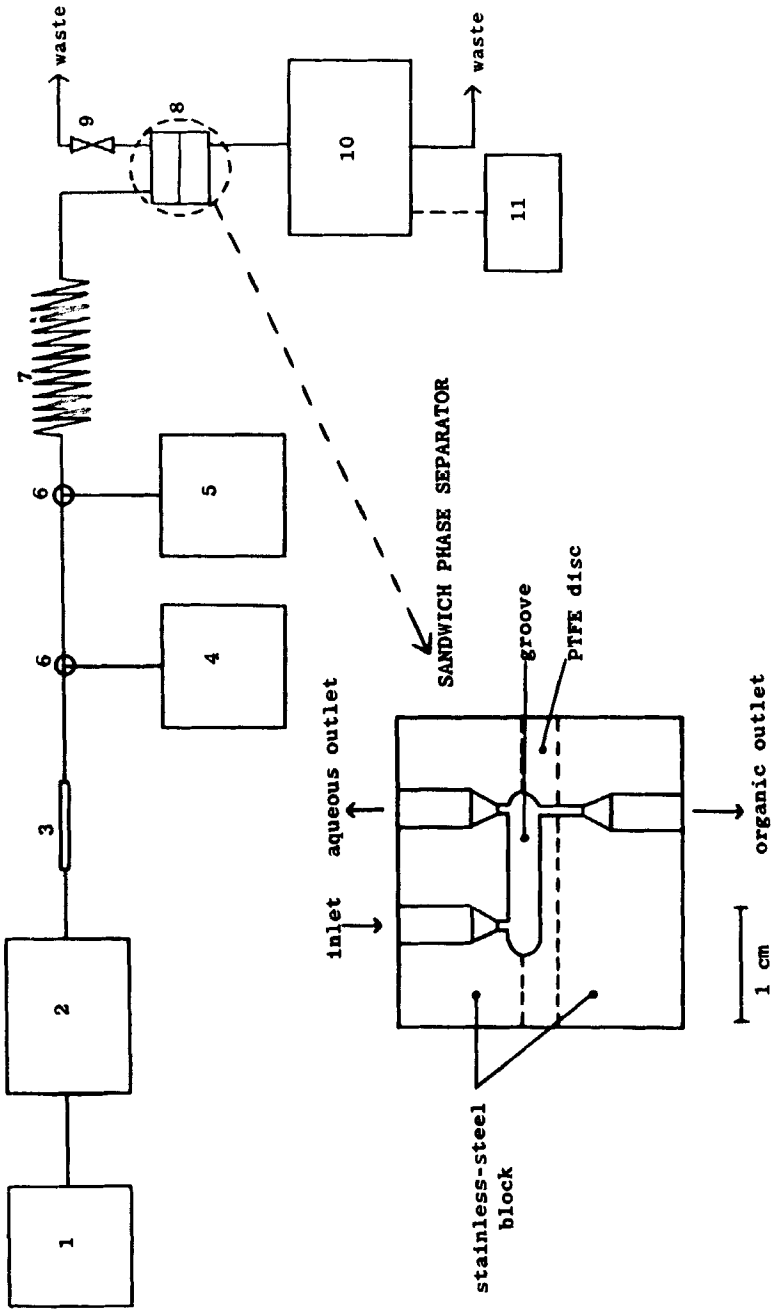


Figure 2.
Block diagram of the automated system. (1) eluent pump; (2) AASP; (3) column; (4) reagent pump; (5) organic extraction solvent pump; (6) T-piece; (7) stainless-steel coil; (8) sandwich phase separator; (9) micro needle valve; (10) fluorescence detector; (11) recorder.

precolumn (with 13 μm frits) which was slurry-packed by means of a micro-spatula with 40 μm Baker C-8 or C-18 material, or using disposable precolumns which were loaded off-line and, next, automatically processed with a Varian (Walnut Creek, CA, USA) advanced automated sample processor (AASP). The disposable precolumns i.e., the AASP cartridges which contain 40-50 mg of 40 μm C-8 packing material, were received as a gift from Analytichem (Harbor City, CA, USA). The 100 x 4.6 mm i.d. LKB (Bromma, Sweden) 3 μm Spherisorb ODS-2 analytical column was a gift from Astra.

The post-column extraction system consisted of two Valco T-pieces with 0.25 mm bores, a 1.5 m x 0.8 mm i.d. stainless-steel capillary (coil diameter, 40 mm) and a home-made sandwich type phase separator (see also Fig. 2). The interior of this separator consists of a groove with a volume of 30-40 μl, the upper half being constructed of stainless-steel and the lower half of PTFE. No membrane is used. With this separator a purely organic phase can be obtained. A detailed description of the design and characteristics of this phase separator will be published in a separate paper [10]. The ratio of the organic flow through detector to the total organic flow was regulated by a SGE (Melbourne, Australia) MCV 50 micro needle valve, connected to the aqueuos outlet of the phase separator. A ratio of 0.30 was the best compromise in terms of signal-to-noise ratio and band broadening.

The eluent used was water-acetonitrile (90:10, v/v) (flow rate 1.0 ml/min) adjusted to pH 3.0 with phosphoric acid, the reagent was a 10^{-4} M DAS solution in water (pH 3.0) (flow rate 0.4 ml/min) and the organic extraction solvent was 1,2-dichloroethane (flow rate 1.0 ml/min).The AASP purge solvent was a 10 mM aqueous sodium phosphate buffer (pH 3.0). For detection a Perkin-Elmer (Norwalk, CT, USA) LS-4 fluorimeter set at λ_{ex} = 383 nm and λ_{em} = 452 nm (slits, 10 nm), was used. Chromatograms were recorded on a Kipp (Delft, The Netherlands) BD-8 recorder.

Analysis of Biological Samples

The home-made 2 x 4.6 mm i.d. precolumn packed with 40 μm Baker C-8 or C-18 material was preconditioned with 5-8 ml of 5.10^{-4} M Cetrimide in water-acetonitrile (65:35, v/v; pH 3.0) and 1 ml of a 10 mM sodium phosphate buffer (pH 3.0). The analytical column was pretreated with 100 ml of 5.10^{-4} M Cetrimide in water-acetonitrile (65:35, v/v; pH 3.0) at a flow rate of between 0.1 and 0.5 ml/min and was subsequently equilibrated with 75 ml of eluent at a flow rate of

1.0 ml/min. Untreated plasma was diluted 1:1 (v/v) with 10 mM phosphate buffer (pH 3.0) and the mixture was injected directly onto the precolumn (total injection volume, 1 ml). After a forward flush wash with 2 ml buffer pH 3.0, the precolumn is switched on-line with the analytical column for 60 s.

Disposable AASP cartridges were preconditioned with 4 ml of the 5.10^{-4} M Cetrimide solution and 1 ml buffer pH 3.0 and loaded with untreated plasma or urine diluted 1:1 and 1:9 with buffer pH 3.0, respectively. The total injection volume was 2 ml. After a wash with 1 ml buffer pH 3.0 the cartridges are transferred to the AASP , where they are processed automatically.

RESULTS AND DISCUSSION

Chromatography of Remoxipride

Reversed-phase LC of Remoxipride as well as other solutes with amine functional groups is troublesome because of interactions between the basic amine function and residual silanol groups present on the silica surface of the packing material [2]. One possibility to solve this problem is the use of an amine modifier as was recently evaluated by Kiel et al. [2] for 15 different modifiers. Unfortunately, the use of these modifiers creates chromatographic systems that are not compatible with post-column DAS extraction detection, because of the capability of the modifiers to form ion-pairs with DAS, which leads to high background fluorescence.

Another approach, i.e., the addition of DAS to the mobile phase and thus chromatography of the Remoxipride-DAS ion-pair, did not give satisfactory results in the present case. Using a 100 x 4.6 mm i.d. Spherisorb ODS-2 column and 10 mM aqueous sodium phosphate buffer (pH 2.5) - 1,4-dioxane (70:30, v/v) containing 10^{-4} M DAS as the eluent at a flow rate of 1 ml/min, the ion-pair had a k' value of about 3, but the peaks were extremely asymmetric ($A_{0.1} = 6$). Another possibility is blocking the residual silanol groups by using a bulky compound that will be stronlgy retained. For that purpose we selected hexadecyltrimethylammonium bromide (Cetrimide). The analytical column was flushed with 100 ml of 5.10^{-4} M Cetrimide in water-acetonitrile (65:35, v/v; pH 3.0) at flow-rates between 0.1 and 0.5 ml/min and equilibrated with 75 ml of eluent (cf. below) at a flow-rate of 1.0 ml/min. Remoxipride does not elute from the ODS-2 column under normal conditions, but after Cetrimide treatment low retention

and excellent peak shapes were observed. In Fig. 3 the relative peak heights of Remoxipride, obtained after loop injections from a standard solution (200 ng/48 µl), are plotted against the total amount of the various eluents flushed through the column. Cetrimide is not stripped off using up to 1000 ml of water-acetonitrile (90:10, v/v; pH 3.0) as eluent, while the use of eluents with a higher (15-20%) acetonitrile content led to a rapid loss in performance of the column. This resulted in lower, broader peaks and, finally, in an increase in retention. Actually, with water-acetonitrile (90:10, v/v; pH 3.0) such phenomena were only observed after 3-4 days, i.e., after the passage of about 2000 ml of eluent. The performance of the column can be completely restored by treatment with the same Cetrimide solution as was used before. Using an eluent containing 10^{-7} M Cetrimide, which is about the maximum concentration compatible with the post-column DAS extraction detector, only slightly increased the period of constant performance of the column.

As a final conclusion, water-acetonitrile (90:10, v/v; pH 3.0) was used as the eluent and the column was treated with Cetrimide every 2 days. In addition to the analytical column, precolumns packed with large particle reversed phase material, also have to be pretreated with Cetrimide in order to prevent strong retention of Remoxipride, which results in extremely broad injection profiles. Using a 2 x 4.6 mm i.d. precolumn, packed with 40 µm Baker C-8 or C-18, 5-8 ml of 5.10^{-4} M Cetrimide in water-acetonitrile (65:35, v/v; pH 3.0) were required for complete coverage of the silanol groups. The disposable 40 µm C-8 AASP cartridges, which contain more packing material, needed only 4 ml of the Cetrimide solution for total coverage. This can be explained by the fact that in the case of the home-made precolumn, the Cetrimide pretreatment is done by means of a syringe whereas the AASP cartridges are more slowly flushed using controlled air pressure. After Cetrimide treatment, the precolumns were flushed with 1 ml 10 mM phosphate buffer pH 3.0 to remove the organic modifier.

Plasma Samples

In the first experiments, preconcentration of 1 ml plasma (untreated plasma - 10 mM phosphate buffer, pH 3.0; 1:1, v/v) was carried out on a 2 x 4.6 mm i.d. precolumn, packed with 40 µm Baker C-8 or C-18. Such a preconcentration step can easily be automated using an autosampler and a column-switching apparatus [11]. Although good chromatograms were obtained, reproducibility based on peak height measurements was rather poor even when the precolumn was repacked after

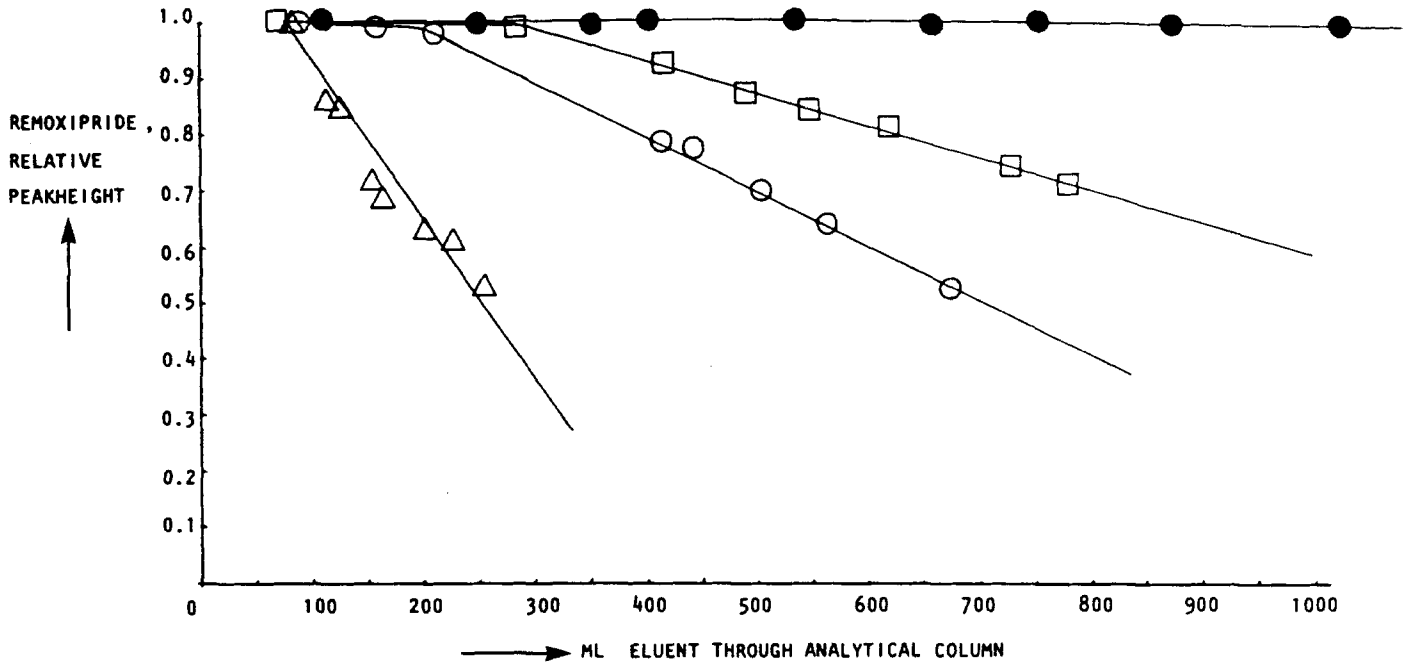


Figure 3.
Dependence of the relative peak height, of repetitive injections of a standard solution of Remoxipride , on the amount of eluent flushed through the Cetrimide-treated analytical column.
(●) Water-acetonitrile (90:10, v/v). (o) Water-acetonitrile (85:15, v/v). (Δ) Water-acetonitrile (80:20, v/v). (□) Water-acetonitrile (85:15, v/v) containing 10^{-7} M Cetrimide. All eluents were adjusted to pH 3.0 with phosphoric acid.

each determination. This observation indicates the necessity of internal standardization. Using FLA 913 as an internal standard, the reproducibility based on Remoxipride/FLA 913 peak-height ratios was good. The overall relative standard deviation for 1 ml preconcentrations of 1:1 v/v diluted plasma, containing 175-200 ng of Remoxipride and FLA 913 was less than 5% (n = 20) within one serial run as well as from day to day. However, two important drawbacks were observed. In the first place, interfering peaks from the plasma matrix were observed after up to 30 min,which is a great disadvantage in routine analysis. Secondly, the precolumns could only be used about three times, even when regenerating them with up to 8 ml of Cetrimide solution after each run. Hence, although column-switching is a viable approach, we preferred to use disposable precolumns in our further experiments.

Disposable AASP cartridges were pretreated with Cetrimide and buffer pH 3.0 and, next, loaded with 2 ml plasma samples (untreated plasma - 10 mM phosphate buffer, pH 3.0; 1:1, v/v) and washed with 1 ml buffer pH 3.0. The use of buffers with a higher pH, i.e., 5-7, immediately resulted in a serious reduction of the recovery. After the off-line sample preparation, the cartridges were automatically processed by the AASP. A pre-injection purge of 1 ml phosphate buffer pH 3.0, which did not result in loss of recovery, was necessary to remove all the air present in the cartridge. The cartridge was then switched on-line with the analytical column for 42 s, which is just long enough to elute Remoxipride from the cartridge, leaving behind all the interfering plasma constituents having a high k' value. Finally, a post-injection purge of 1 ml phosphate buffer pH 3.0, removed the eluent from the AASP connection capillaries before processing a subsequent cartridge. Fig. 4 shows a chromatogram of a duplicate analysis of a plasma sample containing 200 ng Remoxipride per ml. It is clear that only minor interferences from the plasma matrix are present, which suggests that in routine analysis, a determination of Remoxipride in plasma will take about 8 min.

The detection limit in plasma at a signal-to-noise ratio of 3:1, was 1 ng/ml, as can be derived from Fig. 5. The calibration curve of Remoxipride in plasma was linear over at least two orders of magnitude, with a correlation coefficient of 0.9999 (n = 5). The reproducibility of the method, based on peak height measurements for the determination of 200 ng Remoxipride/ml plasma, was good, i.e., 3.5% (RSD) within one serial run (n = 5) as well as from day to day (n = 12). The recovery of Remoxipride from plasma was 88. ± 4 % (n = 3). The use of plasma samples did not markedly affect the performance of the analytical column. After loading a spiked

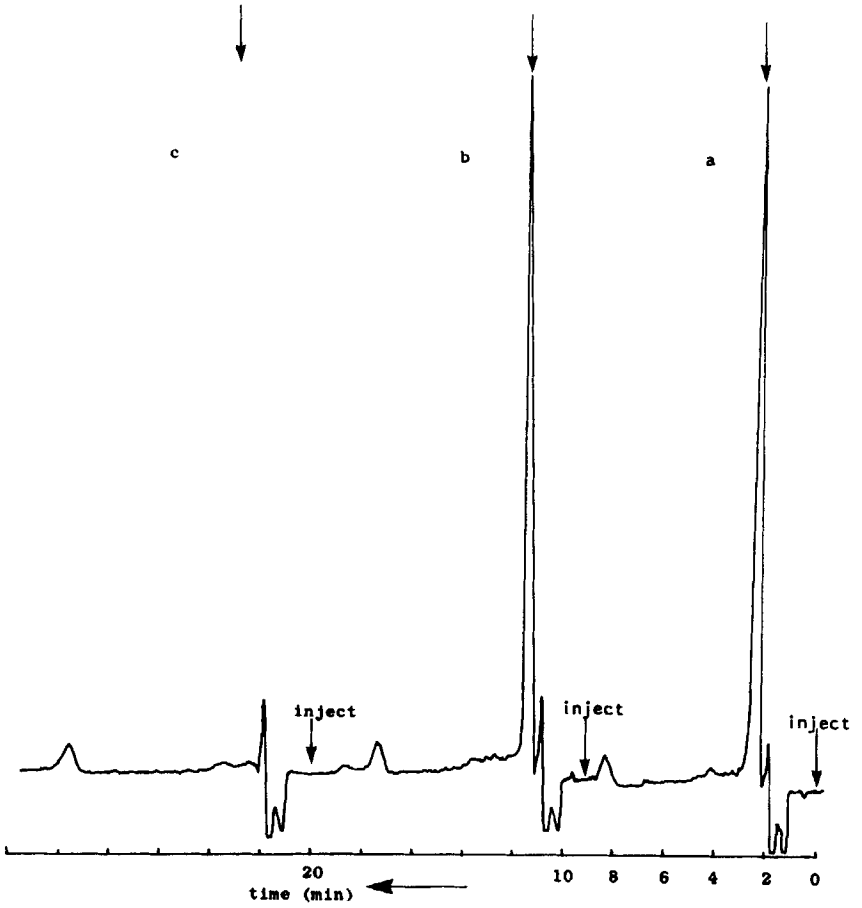


Figure 4.
Chromatograms of plasma spiked with 200 ng/ml Remoxipride (a, b) and of blank plasma (c).

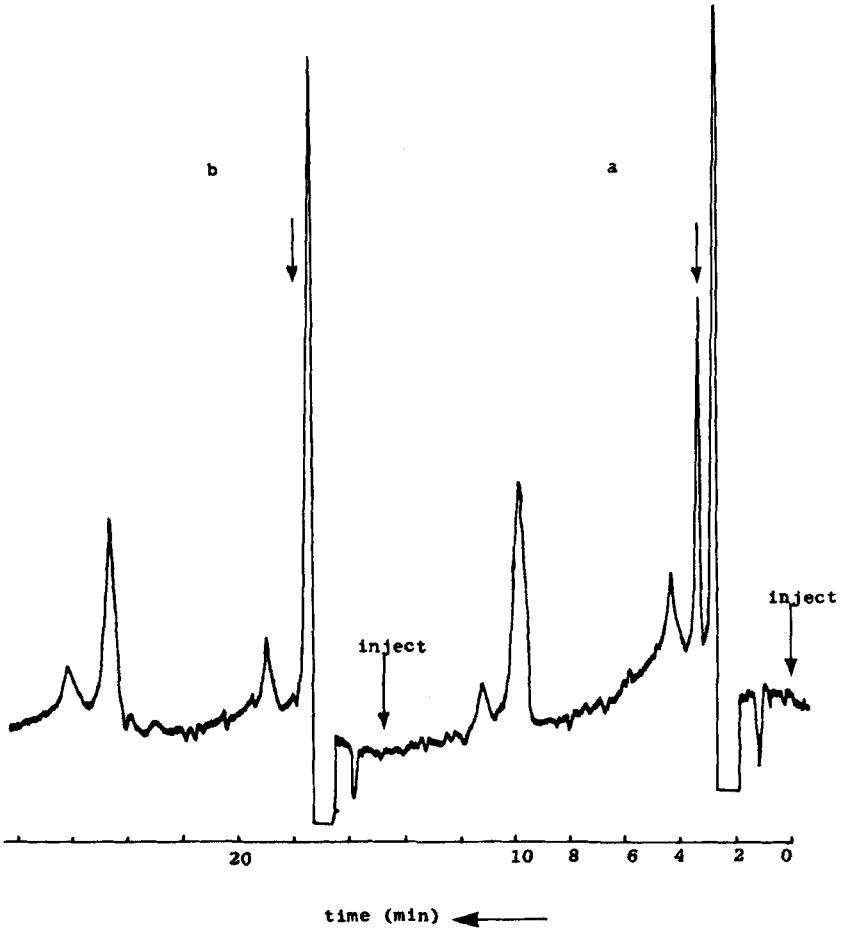


Figure 5.
Determination of the detection limit of Remoxipride in plasma (a) Chromatogram of Remoxipride-spiked (10 ng/ml) plasma and (b) of blank plasma.

plasma sample on the AASP cartridge, the drug is stable at room temperature for at least 20 hours. Taking into account the routine analysis time of 8 min, this means that, the total capacity of the AASP, i.e., 100 analyses could be run overnight without impeding the reliability of the analytical results.

Urine samples

Loading a 1:1 (v/v) diluted 1 ml untreated urine sample on an AASP cartridge leads to an immediate loss of performance, as indicated by the dark yellow-orange colour of the cartridge and the lack of recovery of Remoxipride from the urine sample. Therefore , the amount of urine injected was reduced from 1 ml to 0.2 ml, diluted with 1.8 ml of 10 mM phosphate buffer pH 3.0. The same AASP conditions as described for the determination in plasma were used. Fig. 6 shows a chromatogram of a duplicate determination of 500 ng Remoxipride/ml urine. From the chromatogram it is clear that, although large interfering peaks from the urine matrix are present, a routine determination of Remoxipride in urine can be done in 10 min.

The detection limit of Remoxipride in urine was found to be 15 ng/ml. The calibration curve of Remoxipride in urine was linear over at least two orders of magnitude with a correlation coefficient of 0.9999 (n = 5). The recovery from urine was 76 ± 4 % (n = 3). The reproducibility of the method, based on peak height measurements, was good within one serial run, i.e., 2.2% RSD (n = 4), but was poor from day to day, i.e., 20% RSD (n = 4). An explanation for the latter observation is that interferences from the urine matrix have a negative effect on the performance of the analytical column. As a consequence, the analytical column must be regenerated more often, i.e. 1-2 times every day. One solution to this problem is the use of two parallel analytical columns: while one column is used for analysis, the other is regenerated and treated with Cetrimide v.v.

CONCLUSIONS

A rapid, sensitive and selective method for the determination of the substituted benzamide Remoxipride in plasma and urine, has been developed. The chromatographic behaviour of the drug is strongly improved after treatment of the C-18 analytical column with Cetrimide and when using a mobile phase with a low

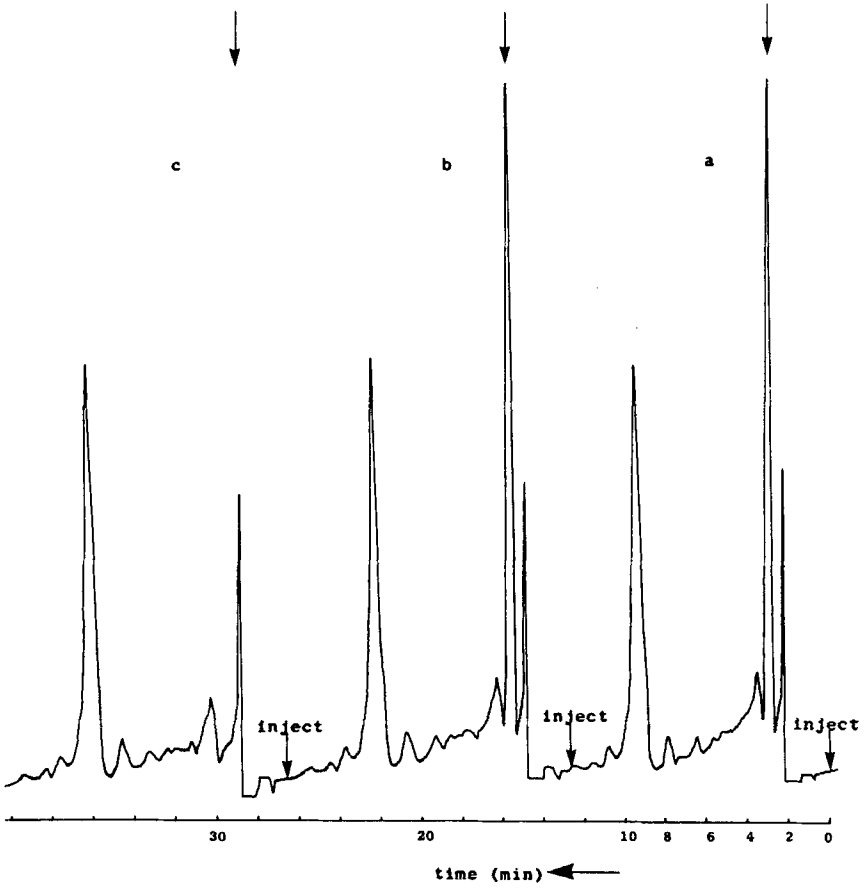


Figure 6.
Chromatograms of urine spiked with 500 ng/ml of Remoxipride (a, b) and of blank urine (c).

amount of organic modifier. Such a system is compatible with post-column DAS extraction detection. This offers great possibilities for the determination of other basic drugs in biological fluids, since the DAS extraction detection system is highly sensitive and selective. Compared to the method of Nilsson [7] where Remoxipride was detected by UV, we achieved a 20-fold improvement in detection limit.

It has been demonstrated that the use of disposable precolumns is recommended in those cases, where regeneration of the precolumn is hard to achieve

and interfering matrix constituents having high k' must be retained on the precolumn to permit short analysis times in routine analysis. A complete automation of the present method may be obtained when the off-line loading of the cartridges is replaced by a procedure involving the use of an autosampler which is capable of loading disposable precolumns [12].

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REFERENCES

1 Ögren, S.O., Hall, H., Köhler, C., Magnusson, O., Lindbom, L.O., Ängeby, K. and Florvall, L., Eur. J. Pharmacol., 102, 459, 1984.

2 Kiel, J.S., Morgan, S.L. and Abraham, R.K., J. Chromatogr., 320, 313, 1985.

3 Lawrence, J.F., Brinkman, U.A.Th. and Frei, R.W., J. Chromatogr., 171, 73, 1979.

4 Lawrence, J.F., Brinkman, U.A.Th. and Frei, R.W., J. Chromatogr., 185, 473, 1979.

5 Reddingius, R.J., De Jong, G.J., Brinkman , U.A.Th. and Frei, R.W., J. Chromatogr., 205, 7, 1981.

6 Wolf, J.H., De Ruiter, C., Brinkman, U.A.Th. and Frei, R.W., J. Pharm. Biomed. Anal., 4, 4, 523, 1986.

7 Nilsson, L.B., Presented at the 9th Int. Symposium on Column LC, July 1-5, 1985 Edinburgh.

8 Bressole, F., Bres, J. and Snousse, M., J. Chromatogr. Biomed. Appl., 343, 443, 1985.

9 Nielen, M.W.F., Koordes, R.C.A., Frei, R.W. and Brinkman, U.A.Th., J. Chromatogr., 330, 113, 1985.

10 De Ruiter, C., Wolf, J.H., Brinkman, U.A.Th. and Frei, R.W., accepted for publication.

11 Werkhoven-Goewie, C.E., Brinkman, U.A.Th., Frei, R.W., De Ruiter, C. and De Vries, J., J. Chromatogr. Biomed. Appl., 276, 349, 1983.

12 Nielen, M.W.F., Valk, A.J., Frei, R.W., Brinkman, U.A.Th., Mussche, Ph., De Nijs, R., Ooms, B. and Smink, W., submitted for publication.